ROLE OF POINT SOURCES ON THE DISSEMINATION OF ANTIBIOTIC RESISTANCE IN THE ENVIRONMENT

A DISSERTATION
SUBMITTED TO THE FACULTY OF
THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Randall S. Singer, Dominic A. Travis (Advisors)

October 2018

Acknowledgements

I would like to first of all acknowledge my advisors, Drs. **Randall S. Singer** and **Dominic A. Travis**. Dominic, you have always been so supportive, even before the PhD when you guided me through my MPH and encouraged me to continue with a PhD. Thank you for all the valuable time you have spent with me and for all I have learned with you. Randy, thank you so much for believing and trusting in me to conduct an international project in Chile. Thank you both for so many learning opportunities, for allowing me to be creative while guiding me when I got off track.

I would also like to thank my committee members (Drs. **Claudia Muñoz-Zanzi, Tim J. Johnson**, and **Nick Phelps**) for their patience and support throughout my PhD. I would also like to give special thanks to **Jessica Williams-Nguyen, Haejin Hwang, André Nault,** and **Jan Sargeant** for their contribution to the development of the two systematic reviews. Furthermore, I would like to thank Drs. **Satoshi Ishii, Qian Zhang** and especially **Britta Wass** and the rest of the Singer lab staff for their help with the molecular laboratory methods. I would really like to thank all the people in Chile that have made this work possible: Dr. **Cristián García**, for his enthusiasm and willingness to cooperate with the trout farms work; Drs. **Paulina Fry, Guillermo Ramirez** and **Keith Hood** (SEREMI de Salud de la Región de los Ríos, Chile) for their help with the wastewater treatment plant work, as well as the staff from the wastewater treatment plant company, especially to Mr. **Jorge Arel**. I would also like to thank the field assistants and staff from the E3 lab at the Facultad de Veterinaria de la Universidad Austral de Chile,

especially Dr**. Claudio Verdugo** for his willingness to collaborate with me and for providing all the field equipment for the bird study. I would also like to acknowledge the investigators from Universidad de Concepcion, especially Drs. **Gerardo González-Rocha** and **Celia Lima** for their support and collaboration. In addition, I would like to thank the faculty, other graduate students, and program coordinators (**Lisa Hubinger** and **Kate Barry**) at the College of Veterinary Medicine (UMN) for their involvement and for creating such a great working environment, and with especial mention to my great UMN friends **Cata, Whitney**, and **Fernanda** who have always been there for me. I could not have got where I am if it was not for Dr. **Patrick Redig**, the champion I followed to Minnesota to learn from, and the staff and volunteers from The Raptor Center, UMN.

On a final personal note, I have to thank all my other numerous great friends, and very especially my best friends, **Ana**, **Paola**, and **Mari**. Also my boyfriend, **Omar** for his patience and love. Finally, my family, "La Piña", especially my brothers **Luis Miguel** and **Jose María** whom I so admire, for their unconditional support and love, as well as **Raluca** and **Ana**, and my adorable and smart nephew, **Robert**, and my niece, **Maria**.

Dedication

Para mis padres Luis y Carmen. To my dad for teaching me that with perseverance, discipline, and hard work I can do anything, and to my mom, la “roca”, for giving me the wings to always follow my dreams. Gracias a los dos por vuestro apoyo incondicional, os admiro y quiero mucho.

Research Abstracts

Chapter 2. Systematic Review: Impact of Point Sources on Antibiotic Resistant Bacteria in the Natural Environment

Point sources such as wastewater treatment plants and agricultural facilities may have a role in the dissemination of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG). To analyze the evidence for increases of ARB in the natural environment associated with these point sources of ARB and ARG, we conducted a systematic review. We evaluated 5,247 records retrieved through database searches, including both studies that ascertained ARG and ARB outcomes. All studies were subjected to a screening process to assess relevance to the question and methodology to address our review question. A risk of bias assessment was conducted upon the final pool of studies included in the review. This paper summarizes the evidence only for those studies with ARB outcomes (n=47). Thirty five studies were at high (n=11) or at unclear (n=24) risk of bias in the estimation of source effects due to lack of information and/or failure to control for confounders. Statistical analysis was used in ten studies, of which one assessed the effect of multiple sources using modeling approaches, and none reported effect measures. Most studies reported higher ARB prevalence or concentration downstream/near the source; however, this evidence was primarily descriptive and it could not be concluded that there is a clear impact of point sources on increases of ARB in the environment. In order to quantify increases of ARB in the environment due to specific point sources, there is a

need for studies that stress study design, control of biases, and analytical tools to provide effect measure estimates.

Chapter 3. Impact of Point Sources on Antibiotic Resistance Genes in the Natural Environment: A Systematic Review of the Evidence

There is a growing concern about the role of the environment in the dissemination of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG). In this systematic review we summarized measurable increases of ARG in the natural environment associated with potential sources of ARB and ARG such as agricultural facilities and wastewater treatment plants. A total of 5,247 citations were identified, including both studies that ascertained ARG and ARB outcomes. All studies were screened for relevance to the question and methodology. This chapter summarizes the evidence only for those studies with ARG outcomes (n=24). Sixteen studies were at high (n=3) or at unclear (n=13) risk of bias in the estimation of source effects due to lack of information and/or failure to control for confounders. Statistical methods were used in 9 studies; 3 studies assessed the effect of multiple sources using modeling approaches, and none reported effect measures. Most studies reported higher ARG concentration downstream/near the source, but heterogeneous findings hindered making any sound conclusions. To quantify increases of ARG in the environment due to specific point sources, there is a need for studies that emphasize analytic or design control of confounding, and that provide effect measure estimates.

Chapter 4. Antibiotic Resistance Genes in Freshwater Trout Farms in a Watershed in Chile

Point sources such as wastewater treatment plants, terrestrial agriculture, and aquaculture, release antibiotic residues, antibiotic resistant bacteria, and antibiotic resistance genes (ARG) into the aquatic ecosystem. However, increases of ARG in the natural environment associated with specific point sources have not been widely quantified. The goal of this study was to evaluate the role of freshwater trout farms on the release and dissemination of ARG into the environment. Sediment samples upstream and downstream from five trout farms were collected over time in southern Chile. A microfluidic qPCR approach was used to quantify an ARG array covering different mechanisms of resistance. Surveys were conducted to obtain information about management practices including antibiotic use at the farms. Florfenicol and oxytetracycline were used at these farms, although at different rates. A total of 93 samples were analyzed. A statistically significant increase of the abundance of *qacG*, *strB*, *sul1*, and several *tet* genes, was found downstream from the farms compared to upstream sites. While results from this study indicate an influence of these trout farms on the presence of ARG in the immediate environment, the biological significance of this finding is unknown and deserves further investigation.

Chapter 5. Role of Wastewater Treatment Plants on Environmental Abundance of Antibiotic Resistance Genes in Chilean Rivers

Point sources such as wastewater treatment plants (WWTPs) commonly discharge their effluent into rivers. Their waste may include antibiotic residues, disinfectants, antibiotic

resistant bacteria (ARB), and antibiotic resistance genes (ARG). There is evidence that ARG can be found in the natural environment, but attribution to specific point sources is lacking. The goal of this study was to assess the release and dissemination of ARG from three WWTPs in southern Chile via two pathways: through the river systems, and through wild birds. A longitudinal study was conducted in small spatial scales around each WWTP to avoid influence of confounders, collecting river sediment samples at different distances both upstream and downstream from each WWTP. Wild birds were sampled from around one of the WWTPs once a month for 13 months. A microfluidic qPCR approach was used to quantify an ARG array covering different resistance mechanisms. There was a statistically significant increase downstream ($p<0.05$) of the abundance of *strB, sul1,* and *sul2*, but the downstream dissemination through the rivers was not clear. Beta-lactamase genes bla_{KPC}, bla_{TEM}, and bla_{SHV} were the most abundant in birds, with higher abundance in migratory species compared to resident species ($p<0.05$). The results from this study indicate that WWTPs in this region of Chile increase ARG abundance in the rivers, but the biological significance of this increase and dissemination further downstream are uncertain. Wild birds were found play a role in disseminating ARG, although association to the WWTP could not be ascertained.

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List of Abbreviations

ARB: Antibiotic Resistant Bacteria

AMR: Antimicrobial Resistance

ARG: Antibiotic Resistance Genes

C_T : Threshold cycle

FAO: Food and Agriculture Organization of the United Nations

MF-qPCR: Microfluidic quantitative polymerase chain reaction

OIE: World Organisation for Animal Health

PACCARB: Presidential Advisory Council on Combating Antibiotic-Resistant Bacteria

WHO: World Health Organization

WWTP: Wastewater treatment plant

Chapter 1. Introduction

Overview

Antimicrobial resistance (AMR) is a global public health challenge. The threat to human health and the associated costs are indisputable. Current international efforts aim at mitigating this threat, but there are still important data gaps that need to be addressed. One area that needs more attention is the dissemination of AMR through the natural environment and the association between environmental levels of AMR and health outcomes. In order to shed light into this important association, the first step is to understand and quantify how AMR gets into the natural environment, how to attribute AMR found in the environment to specific sources, and what are the dissemination pathways of AMR. Even though AMR not only refers to bacterial resistance but also to other microorganisms such as virus and fungus, for the purpose of this dissertation the focus will be on antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG), with special emphasis on ARG. Therefore, from now on, the terms ARB and ARG will be more frequently used than AMR.

This chapter will briefly review the history of antibiotics and AMR, and then it will focus on efforts and challenges to quantify AMR in the environment through the discipline of epidemiology. Note: This chapter include key bibliography related to AMR in general and AMR in the environment, and the remaining chapters include bibliography related to the specific research questions within those chapters. However, literature related to AMR is vast and it is constantly updated, so there might be new studies that were not included here because they were published during the process of submission of this dissertation.

Brief History of Antibiotics and Antibiotic Resistance

Recent history (20th Century)

Prior to the wide use of antibiotics for human medicine in the beginning of the 20th Century, mortality and morbidity caused by infections were very high. From surgical procedures, infected wounds, to diseases such as tuberculosis and syphilis were common causes of death due to lack of effective treatments. The path to antibiotic discovery started in 1904, when Paul Ehrlich and colleagues observed that anilines and other dyes were able to stain some microorganisms but not others. Based on these observations, in 1909 these researchers synthetized an organoarsenic compound derivative that was successful against *Treponema pallidium*, causing agent of syphilis, which was until then untreatable (Thorburn, 1983).

Later, in 1928 Alexander Fleming observed the antimicrobial activity of the mold *Penicillium*, and after experimental trials on animals first and trials on humans second led by Howard Florey with the collaboration of Ernst Chain and Norman Heatley, mass production of penicillin followed during World War II (1939-1945) and thereafter. It was also in the late stages of the War that penicillin was first used by veterinarians to treat bovine mastitis (Gustafson et al., 1997). Around the same time of penicillin discovery, sulfonamides were synthetized by a team from Bayer's laboratory in Germany and were the first antibiotics to be used systemically against streptococci. Another antibiotic, streptomycin, was first used for the treatment of *Mycobacterium tuberculosis*, causing agent of tuberculosis in humans, in 1944. This same antibiotic, a few years later, was observed to improve the growth of chicks when added to their diet (Moore et al., 1946), and the same effect was soon after that observed for other antibiotics and in other animal

groups, such as swine and cattle (Gustafson et al., 1997). After this initial period of antibiotic discovery, it was really during the decades between 1950 and 1970's when the greatest discovery and production of novel antibiotics took place (Aminov, 2010).

Despite the undeniable positive impact of antibiotics on human and animal health, resistant bacterial strains started to be noticed shortly after the first use of penicillin. It was observed that antibiotics exerted a selection pressure on the bacteria, and antibiotics were not effective anymore against those bacteria. When bacteria were in the presence of a selection pressure (e.g. antibiotics), a group of bacteria (resistant) was favored (they survived and multiplied) against another group of bacteria, the susceptible bacteria, which was killed. Sulfonamide resistance was also reported in the late 1930's, and nowadays is actually one of the most broadly disseminated resistance. Resistance of *Mycobacterium tuberculosis* to streptomycin also started to appear, even during a patient's treatment regime (Aminov, 2010, Davies et al., 2010).

Ancient history

Exposure to antibiotics and antibiotic resistance are not a contemporary phenomenon though. Findings of tetracycline traces have been found in the mineral portion of bones in skeletal remains from 350-550 CE. Reports of traces from other antibiotic classes in early civilizations are mostly anecdotal because they are harder to detect, probably due to their different metabolism pathways (Aminov, 2010). Antibiotic resistance is also ancient and it is actually a natural evolutionary phenomenon.

Benveniste et al. (1973) showed that ARG that are now found in clinical settings were originally found in soil microorganisms (Benveniste et al., 1973) and long before antibiotics were used in human medicine, bacteria already carried ARG in nature, as it

has been shown through phylogenetic analysis (Aminov et al., 2007) and by (D'Costa et al., 2011). They analyzed DNA sequences from 30,000 years old permafrost sediment cores and sequenced them, detecting ARG encoding resistance to β-lactams, tetracyclines, and glycopeptides. In another study, soil samples from a remote area in Alaska were collected, and using functional metagenomics, they detected β-lactamase genes that functioned in *E. coli* (Allen et al., 2009).

Despite it being a natural phenomenon, it is without doubt that ARB and ARG levels have increased since the first wide use of antibiotics by humans, around 1940. This increase was evidenced in a study by Knapp et al. where they quantified ARG for different antibiotic classes isolated from DNA collected from archived agricultural soil samples in The Netherlands between 1940 and 2008. Compared to 1970-1979, the relative proportion of resident bacteria containing ARG was 2-15 times higher in 2008. For individual gene copy numbers normalized to 16S rRNA copy numbers, they also found an increase over time for 78% of the ARG quantified, especially for *tetQ, tetO, tetM, bla*$_{TEM-1}$, and *bla*$_{CTX-M-1}$ (Knapp et al., 2009). Even though this study analyzed soils archived from The Netherlands, it is likely that much of the findings could be extrapolated to other parts of the world.

Antibiotic resistance now

The broad use of antibiotics globally for therapeutic uses in humans, animals and crops, and for animal growth promotion during the last 60 years, have enhanced the selection and spread of antibiotic resistant bacteria (ARB) and associated genes (ARG) (Meek et al., 2015, Stockwell et al., 2012, Van Boeckel et al., 2014). Antibiotic resistant pathogens

have a direct negative impact on human health and on animal health and welfare (death, treatment failure, increased costs) (Bengtsson et al., 2014, Friedman et al., 2016).

The human health burden of antimicrobial resistance (AMR) is difficult to estimate; available estimates are usually country-specific. For example, The Centers for Disease Control and Prevention (CDC) has estimated that at least 2 million people become infected with ARB, and at least 23,000 people die every year from these infections in the U.S. (CDC, 2017), and they have listed antibiotic resistant *Clostridium difficile*, carbapenem-resistant *Enterobacteriacea* (CRE), and drug-resistant *Neisseria gonorrohea* (the cause of gonorrhea, a sexually transmitted disease) as the most urgent ARB threats based on level of concern to human health. The animal health burden is even more challenging to estimate than in humans; however, the negative consequences of antibiotic resistance in animals are very similar to those in humans, both for companion animals and for food production animals. There are just a few isolated reports of antibiotic resistant infections with methicillin-resistant *Staphylococcus aureus* (MRSA) in horses and dogs (Catry et al., 2010), as well as infections with methicillin-resistant *S. pseudintermedius* (MSRP) in dogs, which since 2006 has spread globally (Van Duijkeren et al., 2011). In food production animals there are also a few examples such as resistance to penicillin to treat mastitis caused by *Staphylococcus aureus* in cattle (Oliver et al., 2012) or resistance to penicillin and tetracyclines to treat *Pasteurella multocida* and *Mannheimia haemolytica* that cause pneumonia in calves (Portis et al., 2012).

Currently, the problem lies in the excessive use of antibiotics in humans and animals combined with the lack of new antibiotic discovery. Long-term consequences of antibiotic resistance for human and animal health are similar to the current negative

consequences, but it will worsen unless ongoing mitigation strategies are efficacious. A recent analysis conducted by O'Neill modelling six pathogens (*E. coli, Staphylococcus aureus*, *K. pneumoniae*, tuberculosis (TB), malaria and, HIV) predicted that by 2050 more than 10 million people would die from AMR infections per year (O'Neill, 2016). The public health impact of AMR is undeniable, but forecasts like this one have limitations, mostly related to the lack of reliable surveillance data as pointed out by de Kraker et al. (2016) (de Kraker et al., 2016). For animal health, long-term consequences may not only arise from the negative impact of antibiotic resistant infections (treatment failure, mortality), but also from restrictive policies on antibiotic use in animals which will lead to welfare issues and financial losses (Bengtsson et al., 2014).

The global concern over AMR has become an international political issue, and has led to international initiatives involving different agencies (namely WHO, FAO, OIE, and within-region or country initiatives such as Presidential Advisory Council on Combating Antibiotic-Resistant Bacteria in the U.S. or the European Commission Action Plan against AMR) with the common goal of mitigating the risk of AMR to public health. However, mitigating the risk to AMR is a challenging task due to the complexity of the problem which will be reviewed in the following section.

Complexity of Antimicrobial Resistance

In order to understand the complexity of AMR, it is imperative to review a few relevant concepts related to the mechanisms of resistance. It is also important to note that AMR covers resistance to other microorganisms besides bacteria such as virus and fungus (which are out of the scope of this dissertation), and in addition, bacteria (and associated genes) can also harbor resistance to antimicrobials that are not antibiotics (e.g.

disinfectants) and to non-antimicrobials (e.g. heavy metals) through the process of co-selection which is reviewed below.

Mechanisms of antibiotic resistance

There are two main types of bacterial resistance. Bacteria can be naturally resistant to certain antimicrobials (termed intrinsic resistance) which is mediated by chromosomal genes; or they can acquire resistance to antimicrobials to which they were previously susceptible (acquired resistance) (Munita et al., 2016). Either way, bacteria can sometimes become multidrug resistant, which occurs by the accumulation, on resistance plasmids or transposons of genes, each coding for resistance to specific antimicrobials, and/or by the action of multidrug efflux pumps that pump out more than one drug type (Nikaido, 2009). Once bacteria are resistant, they can spread either via vertical transmission (i.e. clonal dissemination) or through horizontal gene transfer (HGT). Horizontal gene transfer occurs via three mechanisms: transformation, which is the acquisition of free DNA from the environment; transduction, which is the transfer of DNA to host bacteria by bacteriophages (virus that infect bacteria); and conjugation, which is the transfer of plasmids (small molecules of DNA within a cell separated from the chromosomal DNA that can replicate independently of the cell) or other mobile genetic elements (MGE) such as transposons or integrons, from one bacterium to another requiring cell-to-cell contact (Henriques et al., 2011) (**Fig. 1**). The genetic exchange through HGT is very relevant in regards to the dissemination of ARB and ARG including through the natural environment, as genes can be exchanged between environmental and clinical bacteria, pathogenic and non-pathogenic bacteria, and even bacteria from different species.

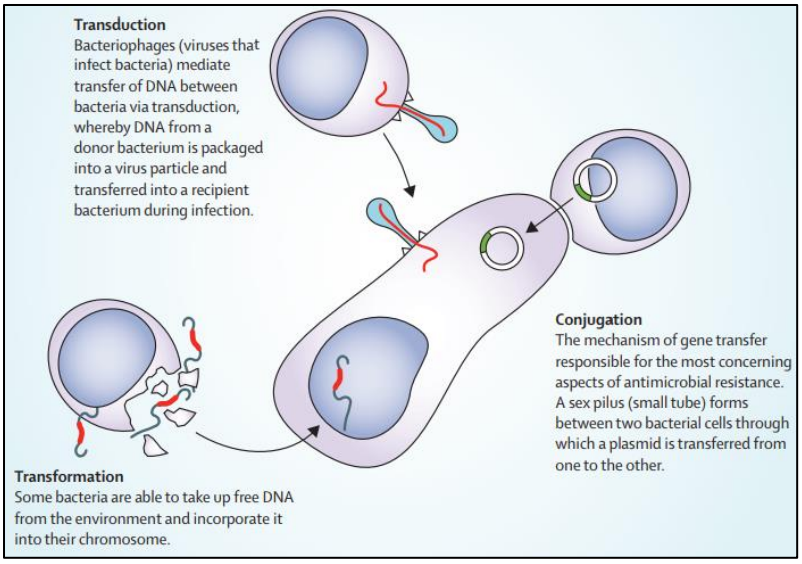


Figure 1. Depiction of the mechanisms of horizontal gene transfer (Figure credit to Holmes et al., 2016).

Against antimicrobials, bacteria use different strategies to thrive in their presence, and these can be grouped into several categories according to the biochemical pathway involved in resistance. It is important to note that bacteria can be resistant to one antimicrobial class through several of these categories: modification of an antibiotic molecule, efflux pump and decreased permeability mechanisms, molecular bypass or changes to target sites, and global cell adaptive processes (Munita et al., 2016).

Modification of antibiotic targets consists of the production of enzymes that change the drug itself making it unable to interact with its target in the bacterial cell. This occurs in Gram-negative and Gram-positive bacteria. An example of this mechanism has been observed for methicillin resistance in *Staphylococcus aureus* (Lambert, 2005). Decreased membrane permeability mechanisms occur when bacteria prevent the antibiotic from reaching an intracellular target by decreasing the uptake of the antibiotic. This is common in Gram-negative bacteria, and affects β-lactams, tetracyclines, and some

fluoroquinolones. Efflux pumps are transport proteins localized and imbedded in the plasma membrane of bacteria capable of ejecting antibiotics out of the cell (Amaral et al., 2014). One example of efflux pumps is resistance to tetracyclines, where *tet* efflux pumps eject tetracyclines out of the bacterial cell (Munita et al., 2016).

Molecular bypass or modification to target sites also affect tetracyclines through the action of *tetM* and *tetO* genes that encode for proteins that protect the target site in the bacteria by either dislodging and releasing the binding of the tetracycline from the ribosome (*tetM*), or by altering the geometry of the binding site for the antibiotic (*tetO*). The last category of resistance mechanisms is global cell adaptive processes which include cell wall synthesis and membrane homeostasis. Resistance to vancomycin in *S. aureus* is an example of this strategy (Munita et al., 2016).

Co-selection

The process of co-selection occurs when there are linked resistance changes among antimicrobial (or even non-antimicrobial) compounds. Co-selection of resistance usually involve biocides such as quaternary ammonium compounds (QAC) and/or heavy metals such as copper or zinc (Wales et al., 2015). The main types of co-selection mechanisms are co-resistance (different resistance genes are located on the same genetic element such as a plasmid, transposon or integron), and cross-resistance (when one biochemical system confers resistance to both antibiotics and metals) (Baker-Austin et al., 2006). An example of both co-resistance and cross-resistance is Methicillin-resistant *Staphylococcus aureus* (MRSA), which include several resistance mechanisms against beta-lactams, macrolides, aminoglycosides and fluoroquinolones (co-resistance), but it also displays cross-

resistance to single antibiotic classes, for example all fluoroquinolones due to mutations in *parC* and *gyrA* genes (Cantón et al., 2011).

Complexity

The intricacies of AMR can be described as a hierarchy with different layers of complexity (**Fig. 2**). There are resistance genes (ARG) that can be exchanged between bacteria (level 1 in the figure) (Frost et al., 2005). These genes can be organized into multidrug resistant bacteria (level 2 in the figure) and are not only able to encode for resistance to antibiotics but also to other compounds through the process of co-selection (Baker-Austin et al., 2006). These two levels were explained in detail in the section above (mechanisms of antibiotic resistance and co-selection).

Further, ARB and ARG can move between different compartments namely human, animal, crops, and the natural environment (level 3). Finally, there is global spread of ARB and ARG (level 4) mostly through short-term travelers, immigrants/long-term travelers, through importation of livestock and agricultural products, and through wildlife movements (Okeke et al., 2001, Choudhury et al., 2012). Despite antibiotic usage being one of the drivers of antibiotic resistance selection and persistence, analyses have suggested that movement of AMR into new areas may be a more important than *de novo* selection (Collignon et al., 2018). One example is the global spread of the plasmid-mediated gene *mcr-1* which encodes for colistin resistance. A phylogenetic analysis was conducted on an assembled global dataset of 457 *mcr-1* positive sequences isolates which established that all *mcr-1* genes that were in circulation descended from a common initial mobilization of *mcr-1* by a transposon in the mid 2000's (Wang et al., 2018). Another example of AMR spread through movement is the increase of bla_{NDM-1} gene abundance

during the month of June in the Upper Ganges River (India), coinciding with the massive movement of people to that area for their religious pilgrimage, compared to gene abundance in the month of February when there is no pilgrimage (Ahammad et al., 2014). As seen in the last example, AMR movement not only applies to global spread, but also to local/regional spread and to spread between the compartments outlined above and further depicted in **Fig 3**. Of these compartments, the natural environment will be the focused of the remaining sections.

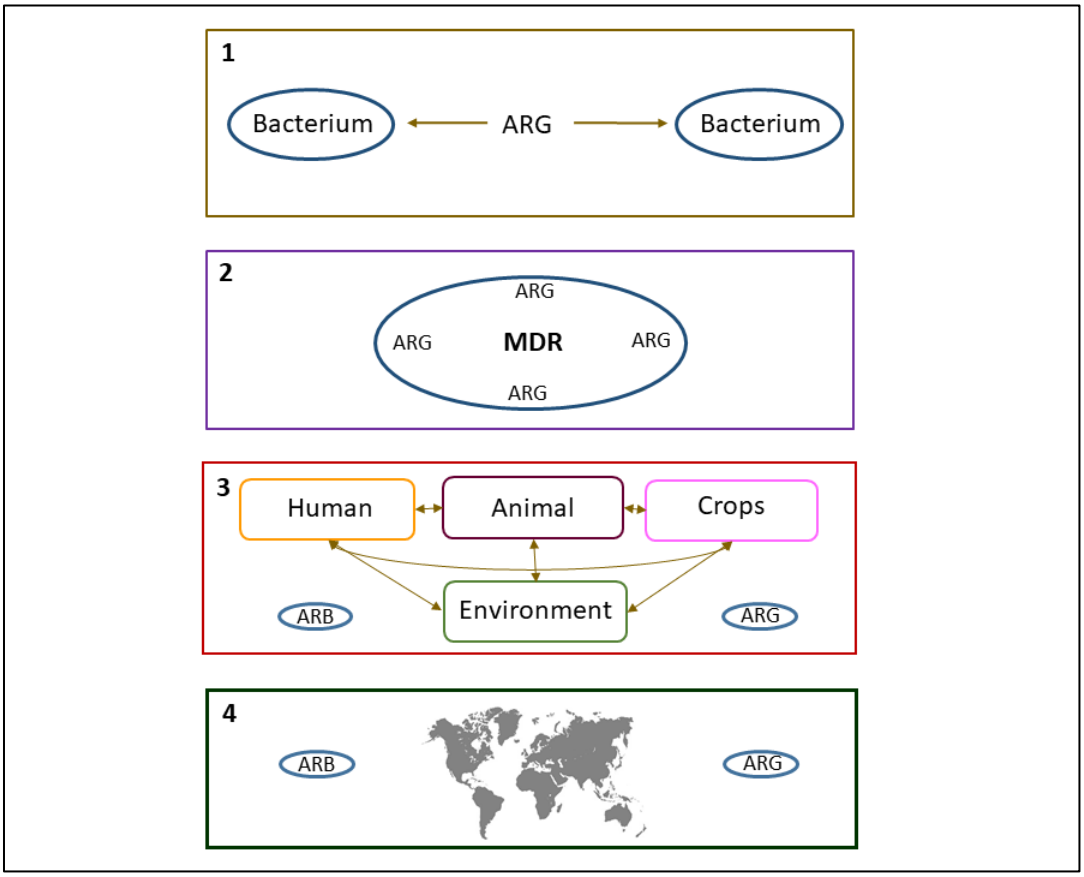


Figure 2. Simplified diagram of AMR complexity.

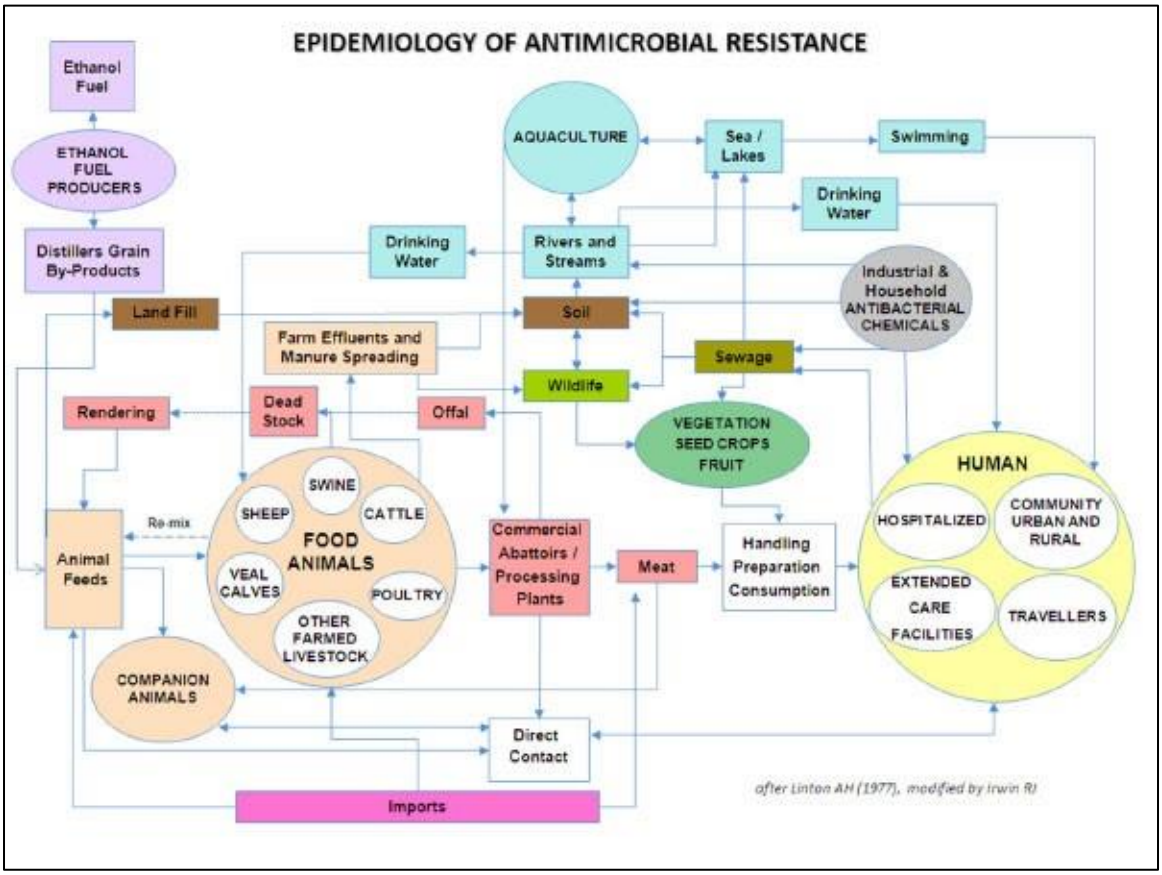


Figure 3. Complex epidemiology of antimicrobial resistance, with human, animals, crops, and the environment as the main compartments and how they are interconnected (Figure credit to Linton AH 1977 and modified by Irwin RJ).

Antibiotic Resistance and the Natural Environment

The natural environment compartment includes surface water (rivers, lakes, streams, wetlands, and oceans), ground water, soil, air, and wildlife. Throughout the literature, the common message is that ARB and ARG are found in the natural environment, in different parts of the world and in a variety of environmental matrices. However, there is still a poor understanding of background levels and increases of ARB and ARG above those levels, attribution of ARB and ARG environmental levels to specific sources; and

what remains the greatest grand challenge is understanding the link between AMR found in the environment and health outcomes, not only in humans, but also in animals and the ecosystem.

Release of antibiotic resistance into the environment

There are a variety of point sources that contribute to the release of ARB and ARG into the environment through their effluents. At the same time, these point sources also release antibiotics and their metabolites, given that humans and animals only partially metabolize antibiotics, so between 30 to 90% are excreted unchanged (Sarmah et al., 2006). Antibiotics (and metabolites) found in the effluent of point sources can select for antibiotic resistance the same way that it may happen in a clinical setting (Allen et al., 2010a). Along with antibiotics and metabolites, other compounds such as disinfectants (e.g. quaternary ammonium), which can also exert selection pressures on the bacteria present at these point sources or on the bacteria in the natural environment, may be released in the effluent as well.

Point sources have been defined as single identifiable sources of pollution, such as a pipe, from which pollutants are discharged (Hill, 2010), and include wastewater treatment plants, pharmaceutical manufacturing effluents, and the effluents from animal operations. There are also non-point sources (i.e. diffuse pollution) such as agricultural runoff that are not discharged from a specific single pipe or drain , and are harder to pinpoint (EPA, 2018) . Non-point sources can also contribute to increases of ARB and ARG in the environment. However, attribution of environmental AMR to a source is more challenging for non-point sources due to their diffuse discharge and impact over larger areas than point sources. Microbial source tracking (MST) has been used in this context

to identify the source of ARB isolated from environmental samples. However, this approach has limitations as it does not provide quantitative estimates of specific impacts. Despite the potential relevance of non-point sources, they are out of the scope of this dissertation, and the main focus remains on point sources.

Pathways of antibiotic resistance dissemination

The role of point sources on the release and dissemination of ARG and ARB into the environment has been closely examined in recent years (Huijbers et al., 2015, Tripathi et al., 2017, Wellington et al., 2013). Waste from animal farms can be in the form of manure (organic matter derived from animal feces) which is often used as fertilizer in agricultural soils. It has been demonstrated that manure can contain ARB and ARG, and thus applying manure to soils is considered a dissemination pathway (Ruuskanen et al., 2016, Xie et al., 2018). Even though soil may be a great microbial reservoir, containing between 10^7 to 10^9 bacteria per gram of soil (Wright, 2010), water is a greater vehicle of bacterial dissemination (Vaz-Moreira et al., 2014). Both ARB and ARG have been found in several aquatic environments (surface water, sea water, groundwater, drinking water) as it has been reported in numerous studies to date (Zhang et al., 2009).

Indirect pathways of ARB and ARG dissemination include human travel and wildlife movements. The number of people travelling internationally nowadays is very large, and this constant movement creates opportunities for ARB and ARG to be acquired by travelers in one country who can carry them later into other countries. There have been studies demonstrating dissemination of ARB via human travel, like the study by Murray et al. (1990) where they collected fecal samples from people before, during, and after visiting Mexico for the presence of resistant *E. coli.* They found an increase in resistance

regardless of exposure to antimicrobials after visiting Mexico (Murray et al., 1990). Based on this and other evidence that was mentioned earlier, it can be concluded that travel and movement of humans, animals or goods are relevant dissemination pathways of ARB and ARG.

Wildlife movements can also present opportunities for ARB and ARG to disseminate. Even though empirical data on the wildlife role of ARB and ARG dissemination is still lacking, there are many studies reporting detection of mostly ARB but also ARG in different wildlife species (Vittecoq et al., 2016). Wild birds are the most widely studied group, mainly due to their ability to migrate across long distances (Bonnedahl et al., 2014, Ahlstrom et al., 2018).

How to Address the Environmental Compartment

In order to further understand key gaps in relation to how ARB and ARG get into the natural environment, attribution of their environmental abundance to specific point sources, and how they disseminate in order to ultimately affect human, animal and ecosystem health, an epidemiological approach that includes adequate study design and analytical methods, as well as laboratory methods to detect and measure ARB and ARG in environmental samples are required. In addition to an epidemiological approach and laboratory methods, international efforts are needed to translate science into policies with the goal of mitigating the risk of AMR in the environment.

Epidemiological approach

Study design

Epidemiology is the field of studying the distribution of diseases in human/animal populations and the factors that cause and influence that distribution (Gordis, 1996).

Epidemiology should be combined with ecology in an eco-epidemiology approach to assess the association between dissemination of ARB and ARG through the environment being released from point sources and health outcomes. The first step in any epidemiological study is to define the specific research question and the system of study (Williams-Nguyen et al., 2017).

After that, one of the main challenges when designing studies is choosing an adequate control group. In epidemiology, the perfect control group would be the exact same site as the one that is under the influence of the point source (i.e. exposed site) but without being exposed to the point source. That way the groups (exposed and control) would be exchangeable. This approach is called counterfactual thinking or potential outcomes model (Greenland, 2005). In the case of the point source assessment, it would involve sampling the sites of interest with and without the point source being there at the exact same time. All the other potential variables would be exactly the same for both sampling events so that the only difference between them would be the presence of the point source. That way, we could say with certainty that the outcome measured (ARB and/or ARG) was caused by that point source because there would not be any confounders or biases to influence the association of interest. In this model, the outcome of interest that is observed is called the factual, and the hypothetical alternative is called the counterfactual (Hernán, 2004). Given this scenario is impossible in the real natural environment, controls for the study would be those sites that are as similar as possible to the impacted (exposed) sites by the point source. In the case that the point source is located in a river, control sites could be upstream sites from the point source, or as suggested by (Downes et al., 2002), better control sites may be parallel rivers to the one

of interest. The ideal way exposed and unexposed (controls) subjects are enrolled in a study is through randomization (allocation of subjects randomly to either exposed or unexposed to make the groups as similar (exchangeable) as possible (Harper et al., 2006). But again, this is impossible to do in the natural environment because a river, lake, or any other natural site can't be randomized to a specific exposure (point source). All of these considerations related to finding the best control group have the ultimate common goal of diminishing the influence of biases (confounding, selection bias, information bias) on the association of interest.

Confounding, as defined by Hernán et al. (2002) occurs when a common cause of both the exposure and the outcome induces a non-causal association between the two factors (Hernán et al., 2002). An example would be the influence of a wastewater treatment plant located immediately upstream from a dairy farm when studying the association of that dairy farm with levels of ARB and ARG in an adjacent river. The wastewater treatment plant would be a confounder if it is also releasing ARB and ARG into the same river and it is spatially close to the dairy farm. In this example, without taking into account the influence of the confounder, it could not be concluded that it was the dairy farm the specific source of ARB and ARG levels in the river.

Selection bias (i.e. collider stratification bias) occurs when a common effect of either the exposure or one of its causes and the outcome or one of its causes influences the inclusion of a subject (in this case a site) in the study (Hernán, 2004). A simplified example of selection bias would be if in river samples downstream from a point source we decided to measure some ARB or ARG while in upstream samples we measured just a few of those ARB and ARG; or if we collected water samples upstream and sediment

samples downstream from the point source and we compared the results between them. We would then be introducing selection bias, because we artificially selected the specific outcomes to measure, or we selected different types of samples across sites.

Finally, information bias (i.e. misclassification) occurs when the measurement on the exposure, the outcome, or any of the other factors included in the study is conducted in different ways so that biases are introduced (or what is called measurement error) (Williams-Nguyen et al., 2017). Again, a simplified example of this type of bias would be if we were to measure ARG in samples from downstream samples using a quantitative PCR (qPCR) approach in one laboratory, while we measured ARG in upstream samples with PCR in a different laboratory and we compared those results.

To minimize these biases, it is important to collect as much data as feasible from factors that could influence ARB or ARG abundance during the design and implementation phases of the study. In the natural environment, there are many variables that may have to be measured across a wide range of spatial scales. To overcome at least part of this challenge, landscape ecology tools have been proposed with this goal (Singer et al., 2006b). Factors to measure and to account for will depend on the point source under study, but they can be physical-chemical parameters, antibiotic use data at the farms or hospitals, among others. However, there will also be variables that will be left unmeasured or that will be unknown. These other potential ways of introducing biases can be at least acknowledged by the researchers when interpreting their results. During the data analysis phase, factors that were measured and that were identified as possible confounders or biases can be included into the statistical models to be accounted for.

Laboratory methods to detect and quantify ARB and ARG

Detecting and quantifying ARB and ARG is an ever changing field, with new tools developing fast. Broadly, these methods can be classified into culture-dependent to detect ARB and culture-independent methods to detect ARG. Culture-dependent methods have been and still are the most important methods to assess antibiotic resistance in clinical settings. Doctors rely on these methods to make clinical decisions about whether an antibiotic is likely to be effective against a bacterial infection (Leekha et al., 2011). In regards to the use of these methods in natural environmental matrices, a recent thorough review has highlighted their advantages and challenges (McLain et al., 2016). Briefly, culture-dependent methods entail the isolation of bacteria on selective media and the posterior assessment of the bacterial growth in response to a determined concentration of antibiotics. After the growth of an isolate in media, confirmation of its identity is recommended either through culture-based or with molecular methods (Peplow et al., 1999).

Target bacteria in environmental samples will depend on the type of sample and system under study, and on the bacterial requirements for growth, but they are usually either pathogens of public health concern and/or bacterial indicators (e.g. *Salmonella spp, E. coli, Aeromonas spp*). Aside from the target bacteria, target antibiotics should cover different antibiotic classes and mechanisms of resistance, and again, should be aimed at the specific sample/s and system under study. Methods to test for ARB following the isolation and identification of an isolate can be broadly classified as broth dilution, and agar disk diffusion methods.

Within broth dilution methods, microdilution is the most commonly used. This method consists of growing the bacteria of interest as a dilution in a 96-well tray that contains the antibiotics to be tested at different concentrations (Reller et al., 2009). This method is used to determine the minimum inhibitory concentration (MIC), which is the lowest concentration of an antibiotic that prevents visible growth of a bacterium. Agar disk diffusion consists of assessing the inhibition zones for the bacteria of interest around antibiotic disks in a Mueller-Hinton agar. E-tests are a combination of the above two methods. Agar diffusion methods provide qualitative results classifying the bacterial isolates as susceptible, intermediate, and resistant, although these results can also be reported as the percentage of isolates that are susceptible or resistant, as well as the zones of inhibition. Usually, larger zones of inhibition correspond to smaller MIC. Minimum inhibitory concentration (MIC) is used to determine antibiotic resistance in clinical settings as the breakpoints inform clinicians of what antibiotic therapy to use against specific bacterial species. But MIC can't be used in other settings such as the environment because environmental bacteria do not have established breakpoints (Martinez et al., 2015).

Advantages of culture-dependent methods are the ability to understand phenotypic features of antibiotic resistance and the confirmation and measurement of living bacteria in the samples. Challenges include knowing what specific strain to target, and biases introduced by culturing methods given that less than 1% of environmental bacteria has been successfully cultured (Ward et al., 1990, Wright, 2010).

Culture-independent methods have the upfront advantage that they do not require culturing the bacterium, but instead their goal is to look for ARG in DNA isolated from

environmental samples. There are several culture-independent methods that have been used for environmental samples, and more details can be found in recent reviews (Henriques et al., 2011, Zhang et al., 2009). The most relevant methods to the work presented in this dissertation are briefly reviewed below.

Polymerase Chain Reaction (PCR), both simple and multiplex, has been traditionally used to detect ARG in DNA, even at low concentrations, from environmental samples. Despite it being fast, reproducible, and highly sensitive method, the main limitation of PCR is that of not being a quantitative method. Quantitative real-time PCR (qPCR) has the advantage over PCR that can quantify ARG. This is done by estimating the initial concentration of the target DNA according to the change of PCR product concentration with amplification cycles (Zhang et al., 2006). Being able to quantify ARG allows researchers to compare ARG abundance between different locations within a study, which includes, for example, assessing how efficacious a treatment technology can be to decrease ARG abundance downstream from a wastewater treatment plant (Sharma et al., 2016).

Microarray techniques can simultaneously quantify multiple ARG in a single assay. Microarrays are high-throughput, fast methods, that require a small volume of reagents and of sample DNA. The most commonly used high-throughput qPCR instruments in the market are: OpenArray (Life Technologies), which uses a chip with 3,072 reactions, each for 33 nanoL reaction volumes, SmartChip (Wafergen), which uses a chip with 5,184 reactions, each for 100 nanoL reaction volumes, and the BioMark™ Dynamic Array (Fluidigm), that combine either 48 samples with 48 assays or 96 samples with 96 assays inside a microfluidic circuit, producing 9,216 reactions in a single run, each for 6.7 nanoL

per reaction (Korenková et al., 2015). Microarrays have been used in clinical settings to detect antibiotic resistance in human pathogens such as *E. coli* or *Salmonella enterica* (Guard-Bouldin et al., 2007, Zhu et al., 2007), but it has been more recently that microarray methods have been applied to ARG detection in environmental samples. For example, water samples collected from different sites in a watershed in China were analyzed using the SmartChip Real-time PCR system (Wafergen Inc. USA) which targeted 285 ARG encoding resistance to 8 antibiotic classes. From the 285 ARG, 258 were detected in at least one sample, being beta-lactams resistance genes the most abundant and higher downstream from anthropogenic activities compared to upstream sites (Wang et al., 2014).

A microfluidic qPCR (MF-qPCR, Fluidigm) array was used to quantify 48 genes that included ARG, heavy metal resistance genes, genes associated with integrons, and 16S rRNA in both treated and untreated wastewater samples, and in drinking water samples (Sandberg et al., 2017). This study was conducted to demonstrate the superiority of MF-qPCR compared to qPCR in quantifying multiple ARG with small sample and reagent volumes, and it also showed that MF-qPCR reduced cost and materials compared to qPCR by more than 95%. Additionally, this paper highlighted the questionable validity of other array approaches that do not include 16S rRNA quantification on the same chip but instead they quantify it using conventional qPCR. The authors claimed that by doing so it is assumed that amplification efficiencies of the genes are the same, which is not likely (Sandberg et al., 2017). The same MF-qPCR method was used to quantify multiple ARG and heavy metal resistance genes in water samples from storm drain outfalls in Florida at different sites and during dry and wet seasons. That study found a variety of ARG

encoding for different antibiotics correlated with sewage-associated markers (Ahmed et al., 2018).

One of the challenges of applying array methods to environmental matrices is their low sensitivity in the sense of their inability to quantify genes that are present in very low copy numbers. However, this issue has been improved by using other methods prior to using the microarray such as specific target amplification (STA) that increase DNA yield (Ishii et al., 2013, Korenková et al., 2015) . Also, environmental matrices are complex in that they may contain contaminants that can inhibit DNA extraction or target gene amplification (Zhang et al., 2009), which is a limitation to acknowledge and that can be avoided by careful handling of the samples and of the laboratory workflow. A general limitation for all these molecular techniques is that they require *a priori* knowledge of primers for target ARG.

Metagenomics, which is the genomic analysis of microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms (Handelsman, 2004), can overcome this limitation because it is not restricted to genes that have *a priori* known primer sequences, and it allows the identification of novel genes in complex samples such as the environment (Pehrsson et al., 2013). Another strength is that it provides both taxonomic and genomic information, including polymorphisms (different variants) present in natural communities. However, disadvantages include difficulty to identify low abundance taxa and to sequence low abundance DNA, as well as lack of availability and access to curated databases across microbial groups (Forbes et al., 2017).

There are other established culture-independent methods, and novel ones such as epicPCR (Emulsion, Paired Isolation and Concatenation PCR). This method recovers

linked phylogenetic and functional information from millions of cells in a single experiment (Spencer et al., 2016). This method was used to link ARG with taxonomic information from influent and effluent samples collected at two wastewater treatment plants in Finland (Hultman et al., 2018). This novel method may be very useful in combining ARG quantification and the phenotypic profile in future AMR environmental studies.

Despite being a very good approach to detecting and quantifying ARG, culture-independent methods also present potential biases that need to be considered and avoided as much as possible. DNA extraction depends on lysis efficiency which varies between microbial groups, thus it could lead to more DNA being extracted from Gram-negative over Gram-positive bacteria or vice versa (Wintzingerode et al., 1997). Also, as pointed out earlier, and despite methods like STA used to decrease this limitation, some of these methods have the challenge of not having enough sensitivity and specificity to detect low levels of ARG in complex environmental samples. Given that both culture-dependent and independent methods provide advantages and disadvantages, a combination of the two should be used to characterize the environment or resistome of a particular system. And overall, provided it is feasible, methods should be used based on the specific research question/s.

International efforts to mitigate antibiotic resistance: One Health approach

One Health has been suggested as an approach to understand AMR complexities given the interconnectedness of the different AMR compartments (Robinson et al., 2016, Van Breda et al., 2017). What this entails is an effective collaboration between the human, animal, and environmental sectors to address and mitigate AMR. This concept has been

incorporated into current international guidelines and action plans with the goal of developing integrated surveillance systems that address AMR in humans, animals and the environment, with a special focus on foodborne bacteria (FAO, 2016, Commission, 2017, OIE, 2017, WHO, 2018). At the moment, the incorporation of AMR measurements as they relate to the environmental compartment are being discussed internationally. At country and regional levels there are also One Health initiatives against AMR like the ones in Europe (Commission, 2018), or the U.S. (e.g. (Health, 2017)). An integrated surveillance system for AMR includes the collection, analyses of trends, and reporting for AMR data across different sectors (animals, crops, the environment), and not just from the public health sectors (WHO, 2017). However, even though we have data from human, animal and environmental compartments from different countries, we still have considerable uncertainty about how to use these data to understand public health and animal health threats.

Summary of the Gaps and Challenges of AMR and the Environment

The environmental AMR compartment is one of the main areas identified by the United Nations international agencies (FAO, WHO, OIE) as well as by scientists requiring more research. For example, Ashbolt et al. (2013) proposed a risk analysis framework to address the main uncertainty of linking environmental AMR to human health outcomes (Ashbolt et al., 2013). More recently, Larsson et al. (2018) reviewed critical knowledge gaps and research needs related to the environmental dimension of AMR (Larsson et al., 2018). These knowledge gaps coincide with those highlighted here before, and include: an understanding of the relative contribution of sources on levels of ARB and ARG in the environment as well as attribution to specific sources; role of environmental sites

impacted by sources on the evolution (mobilization, selection, persistence etc.) and dissemination of ARB and ARG; exposure to humans and animals via different environmental pathways of ARB and ARG dissemination; and ultimately linking it to health outcomes in humans and animals given exposure. An additional gap identified by Larsson et al. (2018) was related to effectiveness of potential mitigation strategies to decrease the dissemination of antibiotic resistance via the environment (Larsson et al., 2018).

Goal of this Dissertation

In an attempt to fill data gaps explained above, the overarching goals of this dissertation were to improve study designs for measuring environmental AMR and to improve our ability to attribute environmental findings to specific point sources. More specifically, the objective was to assess the role of point sources (freshwater aquaculture and wastewater treatment plants) on the release and dissemination of ARG into the Valdivian watershed, southern Chile (**Fig. 4**). In order to assess the role of point sources on the dissemination of ARB and ARG into the environment, the specific aims of this dissertation were as follows:

1. Evaluate and summarize the evidence of the association between anthropogenic point sources and environmental levels of ARB and ARG. For this aim, two systematic reviews were conducted to evaluate methods used to discern increases of ARB and ARG in the environment, and to highlight methodological and research data gaps. Even though both ARB and ARG outcomes were assessed, only ARG were considered for the experimental studies conducted in the dissertation (**Chapters 2, 3**).

2. Assess and quantify the direct release and dissemination of ARG (via water) from freshwater trout farms (**Chapter 4**).

3. Assess and quantify the direct release and dissemination of ARG (via water) and indirect (via wild birds) from wastewater treatment plants (**Chapter 5**).

The long-term goal/broader impact of this research would include the development of a larger scale watershed-based monitoring system to evaluate all point sources in the Valdivian watershed in southern Chile that contribute to the increase and dissemination of AMR. With a better understanding of the dissemination pathways, we would be able to inform strategies to mitigate this risk to public health, animal health, and ecosystem health.

System in Chile

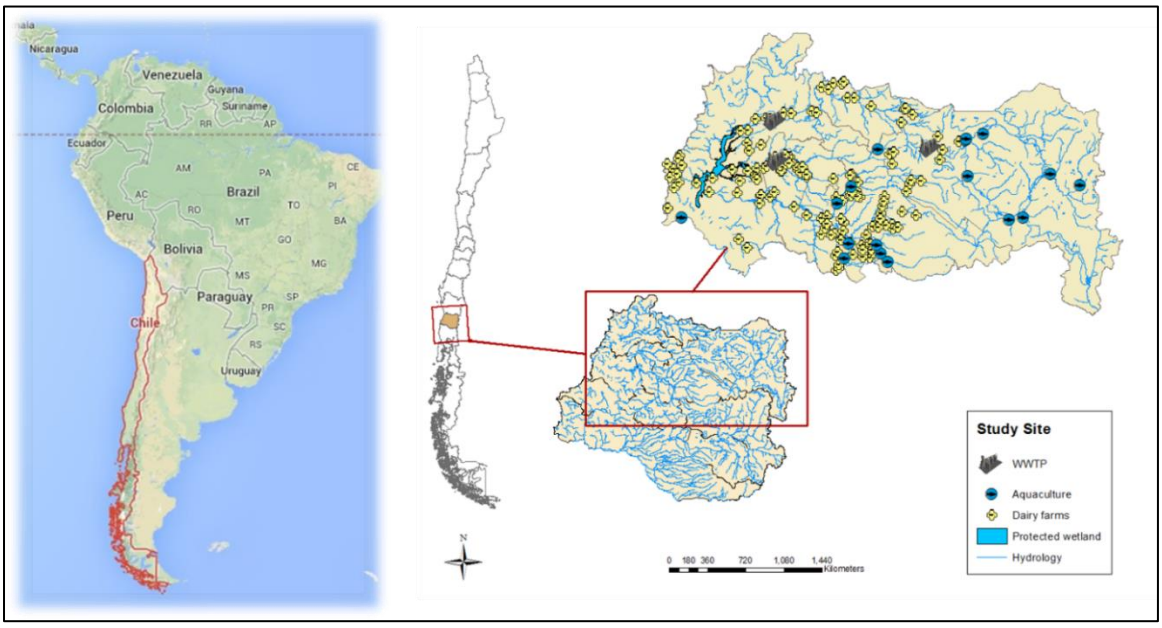


Figure 4. Map of Chile showing the watershed within the Región de los Ríos where the field studies for this dissertation were conducted.

The watershed where the field studies were conducted is located in the Región de los Ríos, southern Chile. This Region borders to the North with the Araucania Region, to the South with Los Lagos Region, to the East with Argentina, and to the West with the Pacific Ocean. The total surface of the Región de los Ríos is approximately 18,429 Km^2 and its population is between 350,000 and 400,000 people, with capital in Valdivia. The climate of this Region is rainy temperate with Mediterranean influence and with influence from its geographical boundaries with both the Ocean and the Andes mountains. The months of June and July have the highest average precipitations. This Region has two main watersheds: the Valdivian and the Bueno watershed. This dissertation was focused on the Valdivian watershed, and its hydrological features have been reviewed before by (del Valle Melendo, 2010).

Briefly, the Valdivian watershed extends 11,320 km^2, 1,048 of which belong to Argentina. The watershed originates in a lake in Argentina, and once in Chile (Pirihueico Lake), it drains through the Fuy River. This river diverges into Panguipulli Lake. From this lake onwards, there is only one river course, but with different names throughout its trajectory, until its outflow into the Pacific Ocean: San Pedro River first, then after receiving effluent from a smaller river, its name changes to Calle-Calle River, and later in the areas nearby Valdivia, the capital, the Calle-Calle receives the effluent from another river, the Cruces River. Where both of these rivers meet, there is an important conservation area, the Carlos Anwandter Sanctuary, designated Ramsar site. Being named Ramsar site means this wetland is of international importance under the Ramsar Convention, an intergovernmental environmental treaty established in 1971 by the United

Nations Educational, Scientific and Cultural Organization (UNESCO) (Ramsar). From that point on, the watershed becomes an estuary until outflowing into the Pacific Ocean.

The main point sources located in this watershed that can release ARB and ARG into the aquatic environment, are mostly human wastewater treatment plants, dairy farms, and aquaculture facilities. There are preliminary studies assessing ARB conducted in this watershed. In a 2-year study, water samples from rivers at upstream and downstream locations from wastewater treatment plants (WWTPs) in the Valdivian watershed were collected. *E. coli* isolates for antibiotic resistance against 16 antibiotics were tested, and the Antibiotic Resistance Index (ARI) was calculated. The prevalence of ARB in the *E. coli* isolates was 30% and 50% in years one and two respectively. In general, there was a higher ARI related to post-treatment (downstream from the WWTPs) (Scherman, 2007). In a 6-week study, water samples from 29 locations at the Cruces and San Pedro rivers were collected. These locations were near either a WWTP (three in total) or a dairy farm, where fecal samples and well water were collected. Results showed higher loads of *E. coli* and ARB levels downstream from the WWTP. However, the *E. coli* and resistance profiles from the dairy farms were not reflected in the river samples taken nearby the farms (unpublished data).

The Valdivian watershed is a source of drinking water, a source of water irrigation for crops, water recreation, and as mentioned earlier, it supports important wildlife conservation areas. Thus the importance to understand the role of point sources on the dissemination of ARB and ARG in this watershed. Using the One Health approach suggested earlier, a workshop with relevant stakeholders was held in 2013 in Panguipulli, (Región de los Ríos) to address water use concerns and ways to study impacts with the

ultimate goal of maintaining a sustainable watershed. Stakeholders present at the workshop included Ministry of Environmental Health, Ministry of Public Health, Ministry of Tourism, Ministry of Indigenous Peoples, Department of Public Works, Servicio Nacional de Pesca y Acuicultura (SERNAPESCA) which is the agency that oversees aquaculture, Arauco (a large timber and cellulose company), Fundación Huilo-Huilo (a private conservation organization based in Huilo-Huilo ecological reserve), Amigos de Panguipulli (a private group of wealthy individuals interested in preserving the rivers in the area), and Academia. Even though this project did not continue due to diverse challenges including lack of funding, this example highlights the importance of communicating and involving all the relevant players in assessing risks in shared environments.

Chapter 2. Systematic Review: Impact of Point Sources on Antibiotic Resistant Bacteria in the Natural Environment

Chapter published as:

Bueno, I., Williams-Nguyen, J., Hwang, H., Sargeant, J. M., Nault, A. J., & Singer, R. S. (2017). Impact of point sources on antibiotic resistance genes in the natural environment: a systematic review of the evidence. Animal health research reviews, 18(2), 112-127.

Introduction

The wide use of antimicrobials, not only in human medicine, but also in livestock, aquaculture, and horticulture, have enhanced the selection and spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) (Baker-Austin et al., 2006, Meek et al., 2015, O'Neill, 2015). When present in human pathogens, antibiotic resistance has a direct negative impact on human health (treatment failure, extended duration of illnesses, death) as well as on the economy (Friedman et al., 2016).

While research and policy have long focused on the role of the built environment such as healthcare facilities, the potential role of the natural environment in the selection and spread of ARB and ARG has been highlighted in recent research (Huijbers et al., 2015) (Hiltunen et al., 2017, Tripathi et al., 2017). Environmental systems, most commonly surface waters, often receive treated and untreated waste from human activities such as households, hospitals, industry and animal agriculture. This waste can contain elevated levels of ARB, ARG, and antibiotics (and/or metabolites) which can then be further disseminated via water, land, or even through wildlife (Baquero et al., 2008, Berendonk et al., 2015, Vittecoq et al., 2016).

The presence of ARB and ARG in the environment likely poses a health risk to humans, animals, and the ecosystem, but this is not well understood (Ashbolt et al., 2013). The first step to assess these risks is to quantify the relative contribution of specific point sources on increases of ARB and ARG in the natural environment. Despite an increase of research in this field, the relative impact of specific types of point sources on levels of ARB and ARG in the environment is still unclear and is an area of ongoing scientific investigation (Williams-Nguyen et al., 2016, Wooldridge, 2012, Woolhouse et al., 2015). The goal of this study was to identify and summarize evidence in the scientific literature pertaining to the association between effluent point sources and the quantity of ARB in adjacent environmental media such as water and soil. We specifically attempted to quantify the strength or magnitude of the effect between a point source (s) and the frequency or concentration of ARB in the surrounding environment. The specific review question was: Is the prevalence or concentration of antibiotic resistant bacteria in soil, water, air or free-living wildlife higher in close proximity to, downstream from or downwind from, known or suspected sources compared to areas more distant, upstream, or upwind from these sources?

Materials and Methods

A systematic review of the literature was conducted following a protocol (Williams-Nguyen et al., 2016) using the population, exposure, comparator, outcome, study design (PECOS) framework. The systematic review team was composed of 6 people, which included expertise on antimicrobial resistance, epidemiology, and systematic review methodology. PubMed©, Commonwealth Agricultural Bureaux (CAB Abstracts©), and Scopus© were searched on October 14th 2014 from inception date using specific search

strategies. The search was updated on April 19th 2016 using identical search terms. The PubMed© controlled-vocabulary search string was as follows:

"drug resistance, microbial"[Mesh] AND ("water pollutants"[Mesh] OR "environment"[MeSH Terms] OR "soil"[MeSH Terms] OR "water"[MeSH Terms] OR "water pollution"[MeSH Terms] OR "air pollution"[MeSH Terms] OR "air pollutants"[MeSH Terms] OR "animals, wild"[MeSH Terms]) AND ("Animals"[MeSH Terms] OR "humans"[MeSH Terms] OR "animal feed"[MeSH Terms] OR "manure"[MeSH Terms] OR "aquaculture"[MeSH Terms] OR "waste water"[MeSH Terms] OR "sewage"[MeSH Terms] OR "hospitals"[MeSH Terms] OR "hospitals, animal"[MeSH Terms] OR "cities"[MeSH Terms]) NOT "therapeutics"[MeSH Terms] NOT "drug discovery"[MeSH Terms] NOT "aids"[All Fields] NOT "hiv"[All Fields] NOT "influenza"[All Fields].

The search string for CAB Abstracts© was:

("Drug Resistance".mp. and ("environment$" or "soil" or "water" or "water pollution" or "air pollut$" or "wild animals").hw. and ("animals" or "man" or "feeds" or "manures" or "aquaculture" or "wastewater$" or "sewage" or "hospitals" or "animal hospitals" or "urban areas").hw.) not "Therapeutics".af. not "Drug discovery".af. not "aids".af. not "hiv".af. not "influenza".af.

The search string for Scopus© was:

TITLE-ABS-KEY ((antibiotic OR antimicrob*) AND resistan*) AND KEY ("environment*" OR "soil" OR "water" OR "water pollution" OR "air pollut*" OR "wild animals") AND KEY ("animals" OR "man" OR "feeds" OR "manures" OR "aquaculture"

OR "wastewater*" OR "sewage" OR "hospitals" OR "animal hospitals" OR "urban areas") AND NOT TITLE-ABS-KEY ("Therapeutics") AND NOT TITLE-ABS-KEY ("Drug discovery") AND NOT TITLE-ABS-KEY ("aids") AND NOT TITLE-ABS-KEY ("hiv") AND NOT TITLE-ABS-KEY ("influenza")

The same protocol was used for both culture-independent (ARG) and culture-dependent (ARB) outcomes, and thus studies with both outcome types were assessed as a whole up to the data extraction process, at which point ARG and ARB outcomes were independently evaluated. More information about studies with ARG outcomes can be found in Chapter 3. There were no language or geographical limits on the search. All citations were imported into the EndNote reference management software package (Thomson Reuters, Philadelphia, PA), and duplicate records were removed.

The first screening stage (relevance screening) consisted of an evaluation of titles and abstracts of all records retrieved to retain only those relevant to the review question. Inclusion criteria required that papers a) were primary research; b) collected environmental samples (soil, water, sediment, air, biological samples from wildlife); and c) reported prevalence or concentration of ARB. An exclusion criterion that was not reported in the original protocol (Williams-Nguyen et al., 2016) was added and asked: "Does the study use microbial source tracking techniques?" Microbial source tracking techniques compare characteristics of fecal bacteria isolated from environmental sources with characteristics of fecal bacteria from known sources in an effort to identify the source of environmental isolates. These types of studies often do not explicitly compare sites based on physical distance or direction from the source (e.g.(Dickerson et al., 2007, Edge et al., 2005, Mthembu et al., 2010, Murugan et al., 2012). As a result, such studies

do not provide evidence for this systematic review question. Any study that did not meet all these criteria was excluded. Those studies for which it was unclear whether criteria were met on the basis of title and abstract passed through to the following screening phase for further review.

Remaining articles were subjected to a second screening stage (design screening) which consisted of an evaluation of the methods section of the full-text to determine if the methodology used for each study was adequate to answer the systematic review question. Inclusion criteria therefore required that studies a) reported proximity to, or direction from a potential point source; and b) had a comparison group (i.e. samples taken a fixed distance from or/upstream from the source) or compared across a range of distances (i.e. samples taken at different distances from the source). Studies that did not meet these criteria were excluded. An additional question not reported *a priori* in the protocol (Williams-Nguyen et al., 2016) was added at this screening stage and asked: "Does the study implicitly or explicitly define a point source to which a comparison is made?" Also, during this screening phase, articles not written in English were identified, and if needed, an effort was made to translate the full-text as the language fluency of the review team allowed.

The systematic review process was pre-tested by reviewing a sample of articles among all the citations from the complete database. Four articles that featured comparison groups based on information in the title and/or abstract were chosen. Studies of this kind were specifically selected to ensure testing of the second screening level (design screening). Two independent reviewers evaluated this phase, and improvements to the screening process and data entry were made based upon their feedback. Records which passed both

screening processes were entered into a spreadsheet designed for this systematic review (Microsoft Office Excel 2013® Microsoft Corporation, Redmond, WA, USA). For both screening phases (relevance and design), two reviewers independently assessed each study. Consensus was required between the reviewers, and conflicts were resolved through phone conferences and e-mail.

After the screening stages, the full-text of each included study was evaluated for potential threats to internal validity (risk of bias assessment) by two independent reviewers. Data for the risk of bias assessment was entered into a customized relational database (Microsoft Access 2013®). First, a qualitative rubric (explained below) was pre-tested by reviewing a sample from the included full-text articles after the two screening stages by three independent reviewers. A total of three articles were evaluated for this purpose. Pre-testing improved the interpretation of the risk of bias assessment across reviewers, as well as the consistency of data entry.

Articles were divided equally between each participating reviewer. A qualitative rubric of low, high, and unclear was assigned to each study for the potential risk of bias in the reported effect measure or other outcome variable due to selection bias, information bias, and confounding (Williams-Nguyen et al., 2016). The risk of bias assessment was conducted at the study level and not at the outcome level due to the large number of possible outcomes per study. Selection bias was defined as systematic differences between the comparison groups with respect to how samples were collected in the study (methods used across sites). Information bias was defined as systematic differences in the methods for ascertaining ARB between comparison groups (i.e. use of different laboratory methods for the samples in the comparison groups). Confounding was

evaluated with respect to the presence of other point or non-point sources that were likely to release ARB, ARG and/or antibiotics to the environment that could have affected the study outcome(s). It was assumed that a study that assessed the impact of a point source using sampling locations within a large spatial scale (e.g. 100 km distance between sampling locations) was at higher risk of confounding than a study where the spatial scale was smaller (e.g. a 10 km scale) due to the possible influence on the outcome of a larger number of alternative point and non-point sources, (and thus release of ARB, ARG and/or antibiotic metabolites) unless adequate confounding control measures were described. For all three types of biases, strategies to control or minimize the impact of these biases on the internal validity of the study were factored into the decision to classify them as low, unclear or high. A final qualitative (low, high, and unclear) overall bias rubric was assigned to each study by considering the risk of bias from each domain after consensus was reached among the reviewers. In general, if a study had at least one of the three domains classified as high risk, the overall result was considered high risk of bias, and the same applied for unclear risk of bias. However, the overall decision was made on a case-by-case basis relying on the judgment of the three reviewers involved in the risk of bias assessment.

After the risk of bias assessment, data from all studies, including those that were deemed to be at high risk of bias, were extracted and synthesized. Data consisted of characteristics of the study (geographic location, publication year, spatial scale, sampling design, type of laboratory detection method used), the exposure (point source), and the outcome: ARB prevalence/proportion or ARB concentration. Any information available on statistical methods or modelling approaches used and effect measures (and variability)

reported for the comparison of interest were also recorded. Data were entered into the same custom relational database albeit in a different table from the one used for the risk of bias assessment. Additionally, a summary of the most relevant findings for the comparison of interest from each individual study was recorded (**Tables 15, 16)**.

In contrast to the original protocol (Williams-Nguyen et al., 2016), the risk of bias assessment was conducted prior to data extraction. To minimize introduction of bias by conducting these steps in reverse order, the reviewers who assessed studies during the risk of bias stage did not review the same studies during data extraction, and were blinded to the risk of bias assessment decisions. Afterwards, a review team member uninvolved in either risk of bias assessment or data extraction validated all the extracted data. A depiction of all the steps that were taken from the protocol until data extraction is found in **Fig. 5**.

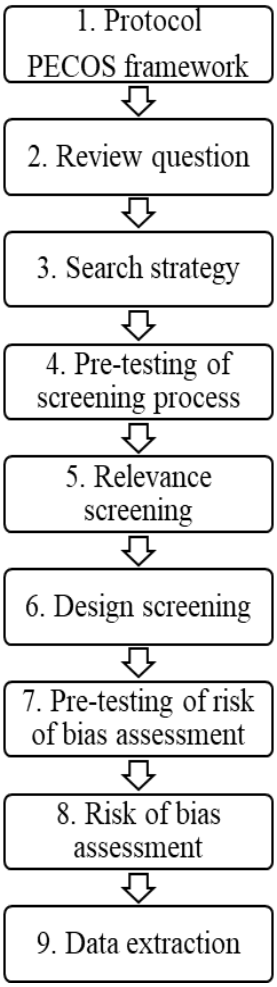


Figure 5. Depiction summarizing the steps of the systematic review process.

Results

Literature searches retrieved a total of 5,247 records after de-duplication. This total included those studies that used both culture-dependent methods to ascertain ARB and culture-independent methods for ARG. After the two screening stages (relevance and design), the number of records retained were 813 (relevance) and 75 (design). Forty-seven of these 75 articles used culture-dependent methods to ascertain ARB (**Fig 6**). The results for ARG outcomes are reported in Chapter 3.

For the overall risk of bias assessment, 11 studies were deemed to be at high risk of bias, 24 were at an unclear risk of bias, and 12 were at low risk for bias. An example of a study considered at high risk of bias was (Laroche et al., 2009). In this study, neither sampling design nor analytic approaches were used to control for potential confounding due to potential point sources other than the wastewater treatment plant (WWTP) of interest that could have influenced the results over the large spatial scale of this study (58 km). Several other WWTPs with potential confounding influence were documented in the study (Laroche et al., 2009). An example of a low risk of bias study was (Harnisz et al., 2015). In this study, sampling was conducted the same way across all sites, the outcome was ascertained consistently at all sites, and the small spatial scale (400 m) made it unlikely for other point sources to influence the results (Harnisz et al., 2015). Finally, an example of an unclear risk of bias study was (Sulzner et al., 2014). In this study, turkey vultures (*Cathartes aura*) were sampled at a location near livestock and at a location far from livestock. The study did not provide enough information to ascertain possible exposure of sampled birds to other point sources of ARB, a plausible issue given that turkey vultures can fly long distances. Also, there was lack of information about sampling procedure consistency between the sites (Sulzner et al., 2014).

Out of the 47 studies, 46 were written in English and one in Portuguese (Fuentefria et al., 2008), which was translated by the review team. The geographic location of the studies was diverse: China (n=7), United States (n= 7), Poland (n=6), France (n=4), Germany (n=4), Spain (n=3), Brazil (n=2), Austria (n=1), Canada (n=1), Croatia (n=1), Ireland (n=1), Japan (n=1), South Korea (n=1), Mexico (n=1), Netherlands (n=1), Portugal (n=1), Romania (n=1), South Africa (n=1), Switzerland (n=1), Taiwan

(n=1), and one study included 2 countries: Saudi Arabia and the UK (Alharbi, 2012). Date of publication ranged from 1995 to 2016, with the highest number of publications in 2015 (n=11). The spatial scale for the sampling frame ranged from 40 meters (Li et al., 2015) to 450 km (Sulzner et al., 2014); six studies did not report a specific distance.

Most studies investigated point sources of human waste (n=35), primarily human wastewater treatment plants (n=26). The remaining studies assessed animal agriculture (n=12), both aquaculture (n=3) and terrestrial agriculture (n=9). Across all studies, surface water was the most common type of environmental media sampled (n=36), followed by sediment (n=5), groundwater (n=4), air (n=2), soil (n=2), biofilm (n=1), wildlife (n=1), and shellfish (n=1). Five studies collected two sample types. Descriptive information about each study can be found in **Table 1.**

Eight of the 47 studies reported more than one bacterium outcome. In those studies where the outcome was detailed at the bacterium species level, the most common bacteria species were: *Escherichia coli (E. coli)* (n=18), *Pseudomonas aeruginosa* (n=3), *Enterococcus faecalis* (n=1), *Enterococcus faecium* (n=1), and *Staphylococcus aureus* (n=1). Some studies only reported the outcome at the genus level: *Enterococcus* (n=5), *Aeromonas* (n=1), *Klebsiella* (n=1), *Pseudomonas* (n=2), or at the family level: *Enterobacteriacea* (n=4) and *Rhodospirillacea* (n=1). Sixteen studies presented their results at a broader level of bacterial classification: heterotrophic (n=8), coliforms (n=5), Gram-negative (n=2), Gram-positive (n=1), *Cytophage-flavobacterium* (n=1), oligotrophic (n=1), and copiotrophic (n=1). Two studies did not specify the bacterial outcome: one reported the proportion of the total resistant bacterial community (Li et al., 2011), and the other one reported oxytetracycline-resistant total culturable organisms

(Kerry et al., 1995). Three studies also reported either the proportion of phenotypically resistant isolates with selected resistance genes (Marinescu et al., 2015, Zhang et al., 2015) or the relative gene abundance among resistant isolates (Hsu et al., 2014).

The ascertainment of ARB was done by disk diffusion in 40 studies, while other methods used in the remaining studies were: dilution method (n=5), and a plating method (n=1). The Clinical and Laboratory Standards Institute (CLSI) guidelines were used for antimicrobial susceptibility in 28 studies, followed by: The French National Guidelines (n=3), Deutsches Institut für Normung or DIN (n=3), Comite´ de l'Antibiogramme de la Societe´ Francaise de Microbiologie or CA-SFM (n=2), defined by the manufacturer (n=2), by the study (n=2), and European Committee on Antimicrobial Susceptibility or EUCAST (n=1). There were five studies where the guidelines were not reported, and one study where the guidelines were not specified.

With reference to statistical methods and modeling approaches, nine studies conducted statistical analysis to compare the prevalence/proportion of ARB between sites with reference to a single point source, and one study used a mixed-model approach to describe the effect of multiple point sources on the proportion of resistant *E. coli* (Rees et al., 2015). None of the studies, however, reported effect measures, which quantify the strength of the effect of the point sources under investigation on the prevalence or concentration of ARB in the natural environment.

For the studies assessing a single point source, (Akiyama et al., 2010, Asfahl et al., 2010) compared the proportion of antibiotic resistant *E. coli* as well as resistant total coliforms between sites upstream and downstream from a WWTP using split-plot ANOVA (Akiyama et al., 2010). Li et al. (2010) compared the prevalence of resistant

heterotrophic bacteria between sites upstream and downstream from a WWTP with a Mann Whitney U test (Li et al., 2010). Schreiber et al., (2013) compared the proportion of resistant *Rhodospirillaceae* between upstream and downstream sites from WWTP using Cramer V correlation (Schreiber et al., 2013). Koczura et al. (2012) compared the proportion of resistant *E. coli* between upstream and downstream sites from a WWTP using a Pearson Chi-square test (Koczura et al., 2012). Sulzner et al. (2014) used this same test to compare the prevalence of resistant *E. coli* in turkey vultures (*Cathartes aura*) between two sites (one near a sheep flock and another site far away from it) (Sulzner et al., 2014). Kotlarska et al. (2015) compared the prevalence of resistant *E. coli* between upstream and downstream sites from a WWTP with Fisher exact test (Kotlarska et al., 2015), and Sapkota et al. (2007) used the same statistical test to compare the proportion of resistant *Enterococcus spp.* at sites upstream and downstream from a swine farm (Sapkota et al., 2007). Fisher's exact test was also used by West et al. (2011) to compare the prevalence of resistant fecal coliforms upstream and downstream from a WWTP (West et al., 2011). Marinescu et al. (2015) compared the proportion of resistant Gram negative bacteria between upstream and downstream sites from a WWTP with one-way ANOVA (Marinescu et al., 2015).

Of these nine studies, six found a significant relationship for some of their comparisons (Akiyama et al., 2010, Koczura et al., 2012, Kotlarska et al., 2015, Li et al., 2010, Marinescu et al., 2015, Sapkota et al., 2007), and three of them did not (Schreiber et al., 2013, Sulzner et al., 2014, West et al., 2011). The study that used a mixed-model approach to describe the effect of multiple point sources on the proportion of resistant *E. coli* in shellfish found that resistant isolates were significantly closer to point sources

compared to non-resistant isolates (Rees et al., 2015). In the section that follows, results are summarized for each group of point sources investigated (human waste and animal agriculture), and by the type of comparison made (upstream vs downstream or upwind vs downwind in unidirectional systems, or based on distance from the source).

Human waste (n=35)

From the 35 studies evaluating human waste, 26 assessed WWTP; three evaluated the effluent from hospitals, three urban areas, and three evaluated sewage sites (one of them defined as a leaky sewer). Among the 26 that assessed WWTP, three compared ARB outcomes based on distance, and the rest (n=23) in unidirectional systems. Of the three studies based on distance, evidence was inconclusive (Czekalski et al., 2012, Kotlarska et al., 2015, Sadowy et al., 2014). Of the studies in unidirectional systems, eight reported higher prevalence/proportion downstream compared to upstream; one study reported higher levels upstream compared to downstream (Zhang et al., 2015); and the remaining (n=14) reported no association or clear trend.

From the three studies that evaluated the effluent from hospitals, two were in unidirectional systems (i.e. rivers) and one was based on distance from the source (hospital). From the two studies in rivers, one reported a higher proportion of resistant isolates downstream compared to upstream (Fuentefria et al., 2008), and the other reported conflicting evidence (Fuentefria et al., 2011). The study based on distance showed no evidence between distance to hospitals and concentration of methicillin-resistant *Staphylococcus aureus* (MRSA) in soil samples (Alharbi, 2012). Of the three studies assessing urban areas, one evaluated the effect of multiple point sources (Rees et al., 2015). This study, based on distance, found that resistant isolates were significantly

greater closer to point sources. The other two studies reported inconclusive evidence for an effect of the urban area on the prevalence of ARB in unidirectional systems (Fincher et al., 2009, Suzuki et al., 2013). One study evaluated the impact of a sewage dump and found higher levels downstream compared to upstream (Goni-Urriza et al., 2000), and one study evaluated a sewage site (Mondragón et al., 2011), reporting inconclusive evidence. One study that evaluated the impact of a leaky sewer on groundwater based on distance found no evidence for increase of ARB levels.

Animal Agriculture (n=12)

Of these 12 studies, three assessed aquaculture and nine terrestrial agriculture. Among the three studies that assessed the impact of aquaculture, two were conducted in unidirectional systems (i.e. rivers), and one was based on distance from a fish farm. Specifically, Harnisz et al. (2015) found inconclusive evidence for the prevalence of resistant heterotrophic bacteria between upstream and downstream sites from a freshwater trout farm (Harnisz et al., 2015a); Gordon et al. (2007) found a higher proportion of resistant *Aeromonas spp.* downstream compared to upstream from freshwater fish farms (Gordon et al., 2007). Kerry et al. (1995) found a higher proportion of resistant bacteria in sites closer to the farms compared to sites farther away (Kerry et al.,1995).

For terrestrial agriculture (n=9), three studies made comparisons based on distance and the remaining (n=6) were done in unidirectional systems. Among the studies based on distance, Li et al. (2015) found no evidence of impact of dairy farms on the prevalence of resistant *E. coli* (Li et al., 2015b)*;* Sulzner et al. (2014) found a higher prevalence (albeit not significant) in turkey vultures closer to a sheep flock compared to a site far away

(Sulzner et al., 2014); and Yao et al. (2011) reported a higher proportion of resistant *Enterobacteriacea* in sites closer to a swine farm compared to sites more distant (Yao et al., 2011). For the studies that made their comparisons in unidirectional systems, von Salviati et al. (2015) reported no difference in the proportion of ESBL between upwind and downwind from swine barns (von Salviati et al., 2015), while Anderson et al. (2014) showed no trend for counts of tetracycline resistant *E. coli* between upstream and downstream sites from a poultry processing plant (Anderson et al., 2014). Laube et al. (2014) showed a potential trend of higher proportion of *E. coli* harboring bla_{TEM-1} downwind from broiler chicken farms compared to upwind samples (Laube et al., 2014). Li et al. (2015) reported higher proportion of ESBL downstream compared to upstream from a swine farm (Li et al., 2015a). Sapkota et al. (2007) reported mixed findings: they found no trend for the proportion of resistant *Enterococcus spp.* in groundwater up gradient and downgradient from a swine farm, but found a higher proportion of resistant *Enterococcus spp.* in surface water in downstream sites compared to upstream sites for most of the antibiotics tested (Sapkota et al., 2007). Finally, Hsu et al. (2014) found an effect of a swine farm on the prevalence of resistant heterotrophic bacteria both in groundwater and surface water (Hsu et al., 2014). Also, among the resistant heterotrophic isolates, they found a possible effect of the swine farm on relative abundance for *sul1* and *sul2* genes. For more details on the results for individual studies refer to **Tables 15** and **16** (human waste and animal agriculture, respectively).

Discussion

The available evidence on the impact of point sources on increases of ARB in the natural environment was evaluated and synthetized in this systematic review. Over the past

decade the number of studies looking at the role of the natural environment on the spread of ARB has increased dramatically. This is reflected in the fact that the highest number of articles that were included in the final pool of this systematic review were published in 2015.

Despite an increase in studies in the last few years assessing ARB in the environment, our specific goal was to identify those studies that were able to measure an effect (or impact) by using ecological or epidemiological approaches/tools. However, most studies were considered to be unclear for risk of bias, primarily because these studies did not provide enough information about potential confounders (especially the introduction of ARB, ARG, and/or antibiotics from other sources in the same system) that could have biased the relationship between proximity to a point source and prevalence/concentration of ARB in the environment. Studies that evaluated sources in a large spatial scale and did not address the influence from additional sources in that scale were most likely to be considered at high risk of bias. With larger spatial scales, there is a more plausible introduction of ARB, ARG, or antibiotics from sources other than the source of interest into the system, which would influence the results. The risk of bias assessment was conducted in a different order from what the protocol had established. However, the authors do not believe this affected or biased the process given that all studies, including the ones considered at high risk of bias, were included in the data extraction, and different reviewers evaluated the same study at the different screening stages. Additionally, although the risk of bias assessment used a qualitative rubric and the choice of bias category (low, unclear, high) was based on reviewer judgement, the quality of

designations was assured by consensus conflict resolution process between the three reviewers.

Wastewater treatment plants were the most common point source evaluated. They have been more extensively studied and have been identified as places of antibiotic resistance emergence due to the favorable conditions and the mixing of bacteria, nutrients and antibiotics (Bouki et al., 2013, Rizzo et al., 2013). However, there are still knowledge gaps and concern about the fate of antibiotics and ARB in WWTP and how that might ultimately impact human health and ecological processes (Kim et al., 2007).

In this systematic review, we found that most studies reported higher levels of ARB in sites downstream from the WWTP compared to upstream sites, or near the WWTP compared to sites far away. However, the evidence documented in this systematic review was primarily descriptive and often did not fully account for possible alternate explanations, and thus it is not possible to quantify the impact of point sources on increases of ARB in the environment.

Studies evaluating animal agriculture as the point source were less abundant than human waste studies. Although animal agriculture (both terrestrial and aquaculture) point sources were documented to disseminate ARB into the natural environment, there was inconclusive evidence of an impact of these sources on increased levels of ARB in environmental media. As antimicrobial use practices change in animal agricultural settings (Directive, 2017), longitudinal studies at consistent sites would be helpful to determine if changes in ARB emission from specific point sources and associated effects on the levels of ARB in the receiving environment can be observed.

Across all studies in the review, resistant *E. coli* was the most frequently studied bacterium. This is not surprising given that these bacteria have been widely studied, some strains have the potential to be pathogenic given their versatility, broad host range, and diversity of virulence factors, and some resistant strains are included among the most relevant antibiotic resistant infections according to the CDC threat list (Blount, 2015, Kaper et al., 2004). Sixteen out of the 47 studies reported their results using very broad categories for ARB outcome (for example, Gram-positive bacteria or heterotrophic bacteria). It is unclear if this was due to the difficulty in identifying the specific bacterium of interest given that less than 1% of bacteria are culturable (Walsh, 2013) or because the bacterial species was not the focus of the study.

As noted in the results, even those studies that conducted statistical analysis did not report effect measures. In some cases, the goal of the studies was not to look at the impact of a point source on the levels of ARB, but just to describe what was found in the environment. However, to gain an understanding of the relationship between point sources and levels of ARB in the environment, it is critical to incorporate ecological or epidemiological tools that permit quantitative estimates of effect. Studying antimicrobial resistance in the environment is very challenging given the complexity of the ecological processes involved, the numerous factors that can affect an observed relationship, and the intrinsic difficulties of field work, to name just a few. To advance the field and improve our understanding of the role of specific point sources on contamination of the natural environment, much more attention must be devoted to careful study design. Then, a combination of statistical methods such as regression analysis, spatial analysis, as well as identification of potential biases and approaches to address those biases to ultimately

report effect measures along with measures of variability should be used. Effect measures are critical to be able to quantify the strength of the effect of the point source (an exposure) on the prevalence or concentration of ARB in the environment (the outcome). Examples of effect measures include odds ratios or risk ratios (for ARB prevalence outcomes) and mean differences (for ARB concentration outcomes).

Given the complexity of studying antimicrobial resistance in the environment, collaboration between ecologists, microbiologists, epidemiologists, soil scientists, hydrologists, civil engineers, agronomists and a variety of other disciplines is needed. Understanding the impact of specific point sources on the levels of ARB and ARG in the environment will be critical to be able to develop effective mitigation strategies to reduce the spread of AMR.

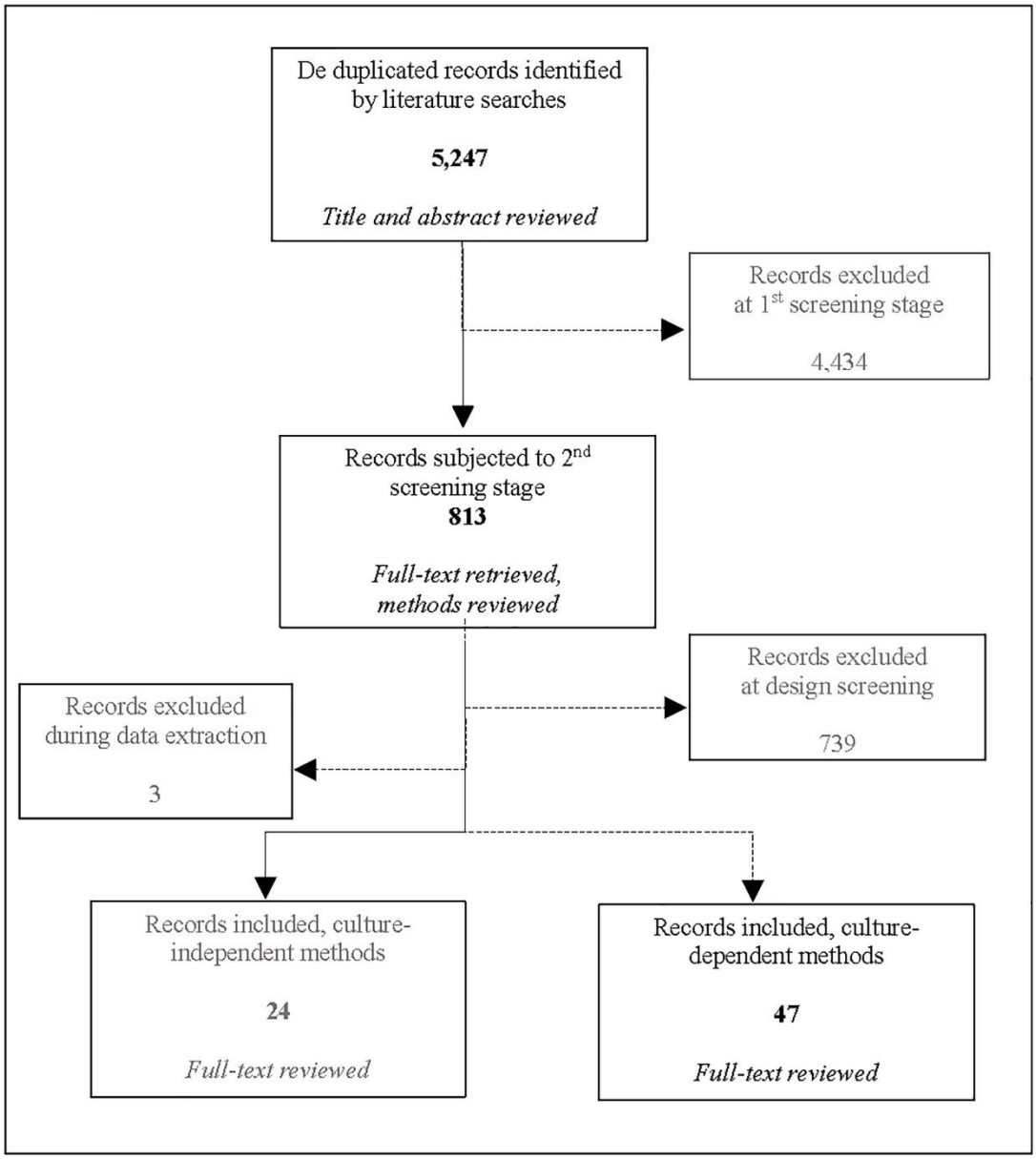


Figure 6. Flowchart summarizing the selection process for the studies included in the systematic review assessing ARB outcomes (the shaded boxes depict the articles excluded from the process and the records for the ARG outcome, not assessed in this chapter).

Table 1. Descriptive information for each one of the 47 studies included in the systematic review assessing ARB outcomes. WWTP: Wastewater treatment plant.

Citation	Country/s	Spatial scale	Source type	Environmental media/System
Abia et al. (2015)	South Africa	50 km	Human waste (WWTP)	Surface water / River
Alharbi (2012)	Saudi Arabia, UK	2 km	Human waste (Hospitals)	Soil
Akiyama and Savin (2010)	United States	2 km	Human waste (WWTP)	Surface water / River
Amador et al. (2015)	Portugal	1 km	Human waste (WWTP)	Surface water / River
Anderson et al. (2014)	United States	0.6 km	Animal agriculture (Poultry processing plant)	Surface water / River
Blaak et al. (2014)	Netherlands	1-2 km	Human waste (WWTP)	Surface water / River
Czekalski et al. (2012)	Switzerland	3.2 km	Human waste (WWTP)	Sediment, surface water / Lake
Fincher et al. (2009)	United States	2-3 km	Human waste (Urban area)	Surface water / River
Fuentefria et al. (2008)	Brazil	Not reported	Human waste (Hospital)	Surface water / River

Citation	Country/s	Spatial scale	Source type	Environmental media/System
Fuentefria et al. (2011)	Brazil	1.4 km	Human waste (Hospital)	Surface water / River
Gallert et al. (2005)	Germany	430 m	Human waste (Leaky sewer)	Groundwater
Goni-Urriza et al. (2000)	Spain	30 km	Human waste (Sewage dump)	Surface water / River
Gordon et al. (2007)	France	2 km	Aquaculture (Fish farm)	Sediment / River
Harnisz (2013)	Poland	2 km	Human waste (WWTP)	Surface water / River
Harnisz et al. (2015)	Poland	400 m	Aquaculture (Fish farm)	Surface water / River
Hsu et al. (2014)	Taiwan	1 km	Animal agriculture (Swine farm)	Groundwater, surface water / River
Kerry et al. (1995)	Ireland	Unclear	Aquaculture (Fish farm)	Sediment / Ocean
Koczura et al. (2012)	Poland	Not reported	Human waste (WWTP)	Surface water / River
Kotlarska et al. (2015)	Poland	3 km	Human waste (WWTP)	Surface water / River
Laroche et al. (2009)	France	58 km	Human waste (WWTP)	Surface water / River

Citation	Country/s	Spatial scale	Source type	Environmental media/System
Laube et al. (2014)	Germany	0.15 km	Animal agriculture (Poultry)	Air
Leclercq et al. (2013)	France	4 km	Human waste (WWTP)	Surface water / River
Li et al. (2009)	China	35 km	Human waste (WWTP treating water from Pencillin G production plant)	Surface water / River
Li et al. (2010)	China	25 km	Human waste (WWTP receiving waste from oxytetracycline production plant)	Surface water / River
Li et al. (2011)	China	35 km	Human waste (WWTP receiving Penicillin G production	Surface water / River
Li et al. (2015a)	China	40 m	Animal agriculture (Swine farm)	Surface water / River
Li et al. (2015b)	United States	Not reported	Animal agriculture (Dairy farm)	Groundwater
Marinescu et al. (2015)	Romania	1.2 km	Human waste (WWTP)	Surface water / River
Marti et al. (2014)	Spain	200 m	Human waste (WWTP)	Biofilm, sediment / River

Citation	Country/s	Spatial scale	Source type	Environmental media/System
Mondragón et al. (2011)	Mexico	Not reported	Human waste (Sewage site)	Surface water / River
Oberle et al. (2012)	France	4 km	Human waste (WWTP)	Surface water / River
Oh et al. (2009)	South Korea	Not reported	Human waste (WWTP)	Surface water / River
Osińska et al. (2016)	Poland	1.2 km	Human waste (WWTP)	Surface water / River
Reinthaler et al. (2003)	Austria	200 m	Human waste (WWTP)	Surface water / River
Rees et al. (2015)	Canada	10 km	Human waste (Urban area)	Shellfish / River
Sadowy and Luczkiewicz (2014)	Poland	37 km	Human waste (WWTP)	Surface water / River, Ocean
Sapkota et al. (2007)	United States	500 m	Animal agriculture (Swine farm)	Groundwater, surface water / River
Schreiber and Kistemann (2013)	Germany	0.16 km	Human waste (WWTP)	Surface water / River
Sidrach-Cardona et al. (2014)	Spain	1.5 km	Human waste (WWTP, Antibiotic-production plant)	Sediment, surface water / River

Citation	Country/s	Spatial scale	Source type	Environmental media/System
Sulzner et al. (2014)	United States	450 km	Animal agriculture (Sheep flock)	Wildlife (Turkey vultures)
Suzuki et al. (2013)	Japan	12 km	Human waste (Urban area)	Surface water / River
Topić Popović et al. (2015)	Croatia	20 km	Human waste (WWTP)	Surface water/ River
von Salviati et al. (2015)	Germany	150 m	Animal agriculture (Swine farm)	Air
West et al. (2011)	United States	2-3 km	Human waste (WWTP)	Surface water / River
Xu et al. (2012)	China	Not reported	Human waste (WWTP)	Surface water / River
Yao et al. (2011)	China	50 km	Animal agriculture (Swine farm)	Soil
Zhang et al. (2015)	China	17.6 km	Human waste (WWTP)	Surface water / River

Chapter 3. Impact of Point Sources on Antibiotic Resistance Genes in the Natural Environment: A Systematic Review of the Evidence

Chapter published as:

Bueno, I., Williams-Nguyen, J., Hwang, H., Sargeant, J. M., Nault, A. J., & Singer, R. S. (2017). Impact of point sources on antibiotic resistance genes in the natural environment: a systematic review of the evidence. Animal health research reviews, 18(2), 112-127.

Introduction

Antimicrobial resistance (AMR) is a serious global public health challenge. Antibiotic resistance in human pathogens can cause treatment failure, prolong the duration of illnesses and increase mortality rates exacting high human and economic costs to society (Friedman et al., 2016). The wide and increasing use of antibiotics and other antimicrobials agents (Baker-Austin et al., 2006) in human medicine, veterinary medicine, animal husbandry, horticulture and around the household have enhanced the selection and spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) to humans, animals, and the environment (Meek et al., 2015, Stockwell et al., 2012, Van Boeckel et al., 2014).

The possible role of the natural environment, and surface water in particular, in transmission pathways of ARB and associated ARG has been the subject of much recent discussion (Wooldridge, 2012, Woolhouse et al., 2015). A range of human activities, including activities of daily living, medical care and agriculture, generate waste that contains varying levels of antibiotics (and metabolites), ARB, and ARG. This waste is ultimately released into environmental media. Point sources, defined as “any single

identifiable source of pollution from which pollutants are discharged" (Armon et al., 2015), represent an important and definable contribution to this effluent stream.

Once in the environment, these ARB and ARG pose potential health risks to humans and animals (Ashbolt et al., 2013). They can persist in the environment, spread over land and water, and be transmitted via free-ranging wildlife. They are also subject to growth processes that can increase their number, in absolute terms or relative to susceptible counterparts. Ultimately, ARB and ARG may be transmitted to humans or animals either as pathogens or commensal organisms. Within environmental niches, ARG can increase clonally when a bacterial cell hosting an ARG divides or be transferred laterally between bacterial cells through horizontal gene transfer (HGT) (Allen et al., 2010b, Ashbolt et al., 2013).

Despite an increase in the number of studies reporting ARB and ARG in diverse natural environmental media, including water, soil, sediment and wildlife, the relative contribution of specific anthropogenic sources to the quantity of ARB and ARG in the environment is an area of debate (Williams-Nguyen et al., 2016, Wooldridge, 2012, Woolhouse et al., 2015). Therefore, in this study we sought to systematically identify and summarize evidence in the existing scientific literature pertaining to an association between effluent point sources and the quantity of ARG in adjacent environmental media. In particular, we looked for measures of impact (i.e. effect measures) which quantify the magnitude or strength of the effect between a point source(s) and the frequency or concentration of resistance elements in the surrounding environment. The specific review question was: Is the prevalence or concentration of antibiotic resistance genes in soil, water, air or free-living wildlife higher in close proximity to, downstream from or

downwind from, known or suspected sources compared to areas more distant, upstream, or upwind from these sources?

Because the majority of bacteria cannot be cultured, many researchers have begun to measure bacterial genes, including ARG, in environmental media using culture-independent methods (Luby et al., 2016). These approaches, such as quantitative real-time Polymerase Chain Reaction (qPCR) and metagenomics (Henriques et al., 2011), are able to provide insight into the environmental resistome in a way not possible using other technologies that rely on culture-dependent methods. Here we report systematic review results pertaining to ARG outcomes (ascertained via culture-independent methods). Culture-dependent methods to ascertain ARB were also reviewed and can be found in a separate publication (Chapter 2).

Materials and Methods

A systematic review of the literature was conducted following a protocol (Williams-Nguyen et al., 2016a) using the population, exposure, comparator, outcome, study design (PECOS) framework. The systematic review team was composed of 6 people, which included expertise on antimicrobial resistance, epidemiology, and systematic review methodology. PubMed©, Commonwealth Agricultural Bureaux (CAB Abstracts©), and Scopus© were searched on October 14th 2014 from inception date using specific search strategies. The search was updated on April 19th 2016 using identical search terms. The search strings were the same as those used for ARB outcomes (Chapter 2).

The same protocol was used for both culture-independent (ARG) and culture-dependent (ARB) outcomes, and thus studies with both outcome types were assessed as a whole up to the data extraction process, at which point ARG and ARB outcomes were

independently evaluated. There were no language or geographical limits on the search. All citations were imported into the EndNote reference management software package (Thomson Reuters, Philadelphia, PA), and duplicate records were removed.

Titles and abstracts of all citations were then screened to include only those relevant to the question. Specifically, studies were included if they a) were primary research; b) collected environmental samples (soil, water, sediment, air, biological samples from wildlife); and c) reported prevalence or concentration of ARB and/or ARG. An additional exclusion criterion -not stated in the original protocol (Williams-Nguyen et al., 2016) - was added that asked: "Does the study use microbial source tracking techniques?". Because these types of studies often fail to compare sites based on physical distance or direction from the source (e.g. (Dickerson et al., 2007, Edge et al., 2005, Mthembu et al., 2010, Murugan et al., 2012)), such studies do not provide evidence for this systematic review question. Any study that did not meet all these criteria was excluded. Those studies where it could not be ascertained from the title and abstract if they met all criteria were considered 'unclear' and passed through to the following screening phase for further clarification.

Full-text of remaining articles was retrieved, and the methods section only was reviewed. It was then determined whether the methodology used for each study was adequate to answer the systematic review question using the following inclusion criteria. Studies were included if they a) reported proximity to, or direction from a potential point source; and b) had a comparison group (i.e. samples taken a fixed distance from or/upstream from the source) or compared across a range of distances (i.e. samples taken at different distances from the source). Those studies that did not meet both criteria were excluded.

An additional question not stated in the protocol (Williams-Nguyen et al., 2016) *a priori* was added at this screening stage as follows: “Does the study implicitly or explicitly define a point source with reference to which a comparison was defined?”. During this screening phase, articles not written in English were identified, and an effort was made to translate the full text as review team resources allowed.

Pre-testing of the screening process was done by reviewing a randomly chosen sample of articles among all the citations by three independent reviewers, and improvements to the screening process and data entry were made based upon the reviewer feedback. Final screening decisions were entered into a spreadsheet designed for this systematic review (Microsoft Office Excel 2013® Microsoft Corporation, Redmond, WA, USA). For both screening phases (title/abstract and methods section of the article’s full-text), two reviewers independently assessed each record. Consensus was required, and conflicts were resolved through phone conferences and e-mail.

Following the application of the inclusion/exclusion criteria, the full-text of each included study was evaluated for potential threats to internal validity (risk of bias assessment) by two independent reviewers per article. A customized relational database (Microsoft Access 2013®) was used for data entry on the risk of bias assessment. First, a qualitative rubric (explained below) was pre-tested by reviewing a randomly chosen sample from the included full-text articles after the two screening stages by three independent reviewers. The pre-testing improved the consistency of the risk of bias assessment across reviewers, as well as the design of the data entry tool.

Articles were randomly allocated to each participating reviewer. A qualitative rubric of low, high, and unclear was assigned to each study for the potential risk of bias in the

effect estimate due to selection bias, information bias, and confounding (Williams-Nguyen et al., 2016). The risk of bias assessment was conducted at the study level, and not at the outcome level due to the large number of possible outcomes per study. Selection bias was defined as systematic differences between the comparison groups with respect to how samples were collected into the study (methods used across sites). Information bias was defined as systematic differences in the methods for ascertaining ARG between comparison groups (i.e. use of different laboratory methods used for the samples in the comparison groups). Confounding was evaluated with respect to the presence of point or non-point sources other than the source of interest that could have affected the study outcomes. It was assumed that a study that assessed the impact of a point source using sampling locations within a large spatial scale (e.g. 100 km distance between sampling locations) was at higher risk of confounding than a study where the spatial scale was smaller (e.g. a 10 km scale) due to the possible influence on the outcome of a larger number of alternative point and non-point sources, unless adequate confounding control measures were described. For all three types of biases, strategies to control or minimize the impact of these biases to the internal validity of the study were factored into the decision to classify them as low, unclear or high.

A final qualitative (low, high, and unclear) overall bias rubric was assigned to each study by considering the risk of bias from each domain after consensus was reached between the reviewers. In general, if a study had at least one out of the three domains classified as high risk, the overall result was considered high risk of bias, and the same applied for unclear risk of bias. However, there were exceptions, and the overall decision was made

on a case-by-case basis relying on the judgment of the three reviewers involved in the risk of bias assessment.

Data from all studies, including the ones that were deemed to be at high risk of bias, were extracted and synthesized. Data consisted of characteristics of the study (geographic location, publication year, spatial scale, sampling design, type of laboratory detection method used), the exposure (point source), and the outcome: ARG prevalence or ARG concentration (either relative gene abundance, -defined as ARG copies normalized to 16S rRNA copies- or absolute gene abundance or gene concentration– defined as ARG copies divided by a measurement of volume), as reported by the authors, without further manipulation of that data. Any available information on statistical methods or modelling approaches used, and effect measures (and variability) reported for the comparison of interest were recorded. Data were entered into the same custom relational database albeit in a different table from the one used for the risk of bias assessment. Additionally, a summary of the most relevant findings for the comparison of interest from each individual study was conducted and it is captured in **Tables 17, 18**.

In contrast to the original protocol (Williams-Nguyen et al., 2016), the risk of bias assessment was conducted prior to the data extraction. To minimize introduction of bias by conducting these steps in reverse order, the reviewers who assessed studies during the risk of bias stage did not review the same studies during the data extraction, and were blinded to the risk of bias assessment decisions. Afterwards, a review team member uninvolved in either risk of bias assessment or data extraction validated all extracted data.

Results

The total number of records (including both culture-dependent to ascertain ARB and culture-independent methods for ARG) returned by search strings totaled 5,247 after de-duplication. The number of articles remaining after the two screening steps was 813 and 75, respectively. Twenty seven of the 75 included articles used culture-independent methods to ascertain ARG. At the point of data extraction 3 studies were identified wherein data were presented as aggregated and no qualitative or quantitative comparison of ARG prevalence or ARG concentration by distance or direction from the source was available. Therefore, these studies were excluded as providing no information about this systematic review question (Auerbach et al., 2007, Bajaj et al., 2015, Xi et al., 2015). Hence, the final number of studies assessed in this review was 24. Three of these 24 manuscripts also reported ARB outcomes, ascertained by culture-dependent methods. The results for the ARB outcomes of those publications along with the rest of the ARB results are presented in Chapter 2 (**Fig 7**).

For the overall risk of bias assessment, 3 studies were categorized as high risk of bias, 13 were at an unclear risk of bias, and 8 were deemed to be at low risk for bias. An example of a study considered at high risk of bias was Zhang et al. (2013). This study collected samples at a spatial scale of about 50 km, and did not adjust for potential confounders in the analysis, such as other point or non-point sources in the 50 km study area (Zhang et al., 2013). An example of a low risk of bias study was Pruden et al. (2012). Despite a spatial scale of more than 100 km, this study controlled for potential confounders from many other sources of anthropogenic effluent by using linear regression modeling to account for distance to different source types (Pruden et al., 2012). An example of a study

with unclear risk of bias was Lapara et al. (2011). In this study, description on the selection of samples at different locations was lacking. Additionally, there were possible confounders such as effluent from non-point sources from agricultural and recreational water use that were not mentioned. Given the lack of information, it was not possible to determine if the risk of bias of estimates of the relationship between the source of interest (WWTP) and ARG concentration (intl1, *tetA*, *tetX*, and *tetW*) in river and lake surface waters and sediments was high or low in this study, and thus it was classified as unclear (LaPara et al., 2011).

While all included studies were written in English, one study written in Chinese (Liu et al., 2012) was deemed relevant to the review question based on the title and abstract that were available in English; however, full-text translation was not feasible, hence it is uncertain if it would have been finally included.

The geographic location of the studies (n=24) was diverse: China (n=3), Finland (n=1), India (n=1), Pakistan (n=1), Poland (n=2), Spain (n=4), Sweden (n=3), Switzerland (n=2), United Kingdom (n=1), United States (n=7). There was 1 study (Stalder et al., 2014) in which the location could not be ascertained after reviewing the full-text, and 2 studies involved 2 different countries (Tamminen et al., 2011, Kristiansson et al., 2011). Date of publication ranged from 2006 to 2016, with the majority published in 2015 (n=7). The spatial scale for the sampling frame ranged from 10-20 m (McEachran et al., 2015) to more than 900 km (LaPara et al., 2015b).

The majority of studies investigated point sources of human waste, especially wastewater treatment plants (n=16), but also cities (n=3). Terrestrial animal agriculture was examined in 3 studies: 2 studies examined swine farms and 1 study examined beef cattle

feedlot. Aquaculture (fish farms) was assessed in 2 studies. Surface water was the most common type of environmental media sampled (n=13), followed by sediment (n=12), biofilm (n=2), air (n=1), and groundwater (n=1). None of the included studies sampled wildlife. Five of the studies collected more than one sample type. For a summary of the sampling information, see **Table 2**.

Overall, the most common target gene outcomes were *sul1* (n=12), *tetW* (n=11), *tetA* (n=9), and *sul2* (n=8). The number of genes per study ranged from 1 to 13, with the majority of them evaluating 4 different genes (n=7). Most studies used qPCR to ascertain ARG (n=23), and 1 study used shotgun metagenomics (Kristiansson et al., 2011). Regarding outcome data type, 20 studies compared relative gene abundance only, 3 compared absolute gene concentration only, and 1 study compared both relative gene abundance and absolute gene concentration. None of the studies used prevalence as their outcome type.

With reference to statistical methods and modeling approaches, 9 out of the 24 studies conducted statistical analysis to compare ARG outcomes upstream versus downstream (or near versus far sites) with reference to a single point source, and 3 out of the 24 studies used modeling approaches to describe the effect of multiple sources. However, no effect measures were described in any study. Specifically, one study used a t-test to compare relative gene abundance of each one of the target ARG between upstream and downstream sites from a WWTP (Berglund et al., 2015). Eight studies compared the relative gene abundance (Harnisz et al., 2015, Khan et al., 2013, Korzeniewska et al., 2015, Marti et al., 2013, Makowska et al., 2016, Proia et al., 2016, Stalder et al., 2014), or the absolute gene concentration (Rodriguez-Mozaz et al., 2015, Uyaguari et al., 2011)

across sites using either ANOVA or a non-parametric method for comparison of means such as Kruskal-Wallis, Friedman, or Mann-Whitney tests at the 0.05 significance level. One study compared the relative gene abundance of ARG across sites based on distance from the source using graphical regression and interpolation (Czekalski et al., 2014). Of the 9 studies that reported statistical inference, 6 found a significant relationship for the majority of the target ARG (Berglund et al., 2015, Khan et al., 2013, Marti et al., 2013, McEachran et al., 2015, Proia et al., 2016, Uyaguari et al., 2011) and 3 did not (Harnisz et al., 2015b, Makowska et al., 2016, Stalder et al., 2014). Of the 3 studies that conducted modeling approaches, (Amos et al., 2015) used a log-log regression model to explain the relative abundance of *intl1* in river sediment samples at sites across a range of WWTP outputs, adjusting for other variables; (LaPara et al., 2015a) used a fluid-kinetics (plug-flow) model to explain the relative abundance of ARG in a river as a function of several variables, including distance from the multiple WWTP; and (Pruden et al., 2012) conducted general linear regression models to explain the log relative gene abundance along a river with an exposure gradient as a function of several variables.

In the section that follows, results are summarized for each group of point source investigated (human waste and animal agriculture) by the type of comparison made (unidirectional systems or based on distance from the source) and by type of outcome reported (relative gene abundance or absolute gene concentration).

Human waste (n=19)

From the 19 studies, 16 assessed WWTP and/or industrial waste, and 3 cities. Among the 16 that evaluated WWTP and/or industrial waste, 13 compared ARG outcomes in unidirectional systems (n=10) or based on distance (n=3) with reference to a single point

source, while 3 studies described the effect of multiple point sources using modeling approaches. Among the 10 studies that assessed the impact of WWTP and/or industrial waste in unidirectional systems (i.e. rivers), 8 of them reported relative gene abundance only, one reported absolute gene concentration only, and 1 reported both. Among the first group, 4 studies showed a higher relative gene abundance at downstream sites from the source compared to upstream sites (Berglund et al., 2015, Kristiansson et al., 2011, Makowska et al., 2016, Proia et al., 2016). One study reported no difference in relative gene abundance downstream compared to upstream (Stalder et al., 2014). The remaining 3 studies presented conflicting evidence for the effect of WWTP/industrial waste on the relative gene abundance (Marti et al., 2013, Sidrach-Cardona et al., 2014, Xu et al., 2015). Regarding absolute gene concentration only, the one study presented conflicting evidence (Rodriguez-Mozaz et al., 2015). Finally, Uyaguari et al. (2011) reported both a lower gene concentration and a lower relative gene abundance downstream (Uyaguari et al., 2011).

Three studies assessed the impact of WWTP across a range of distances. Two of them reported relative gene abundance and 1 study gene concentration. Among the former group, 1 study found higher relative gene abundance at sites closer to the source compared to distant sites (Czekalski et al., 2014) and the other study found no difference in relative gene abundance between near and far sites (Czekalski et al., 2012). The study reporting gene concentration found higher gene concentration at sites closer to the source compared to distant sites (LaPara et al., 2011). The remaining 3 studies assessing the impact of WWTP conducted modeling approaches and they all reported relative gene abundance. The model conducted by (Amos et al., 2015) indicated that a 10% increase in

the total WWTP impact (defined as a function of type of, size of, and river course distance from upstream WWTPs) at a given site was associated with a 3.2% increase in the relative abundance of intl1 adjusting for land cover, season, and rainfall. The fluid kinetics model predictions by (LaPara et al., 2015a) for the Mississippi river did not show a good fit for the target genes, and the general linear regression models in (Pruden et al., 2012) in a river system in Colorado showed an association between average log relative *sul1* abundance and the impact of inverse-distance weighted upstream WWTP and animal feeding operation capacities; however, they did not find such an association for the other target gene (*tetW*).

The 3 studies that assessed the impact of cities as the source of human waste reported relative gene abundance in river systems. Khan et al. (2013) found a higher relative gene abundance downstream compared to upstream sites (Khan et al., 2013); Zhang et al. (2013) found no difference between upstream and downstream sites from a city (Zhang et al., 2013); and Pei et al. (2006) reported mixed evidence depending on the sampling season (high vs low water flow) and on the target genes (Pei et al., 2006).

Animal Agriculture (n=5)

Of these five studies, 3 of them assessed terrestrial agriculture and 2 aquaculture. Among the 3 studies that assessed the impact of terrestrial animal agriculture, 2 were conducted in unidirectional systems (i.e. rivers), of which 1 reported relative gene abundance, and 1 absolute gene abundance. Specifically, (McEachran et al., 2015) reported a higher relative gene abundance downwind compared to upwind sites from beef cattle feedlots; and (Hong et al., 2013) did not find a difference in absolute gene abundance between upstream and downstream sites from a swine farm. The third study examining terrestrial

animal agriculture made comparisons based on distance from a swine farm, reporting higher relative gene abundance near the farm compared to sites farther away (Jia et al., 2014).

The 2 studies assessing aquaculture as the point source made comparisons of relative gene abundance in a river system (Harnisz et al., 2015) and based on distance from the fish farm (Tamminen et al., 2011). Harnisz et al. (2015) found a higher relative abundance of some target genes downstream compared to upstream sites from a fish farm depending on the sampling season, while Tamminen et al. (2011) did not find an apparent impact of the fish farm across a range of distances on the relative gene abundance. For more details on the results for individual studies refer to **Tables 17** and **18**.

Discussion

This systematic review aimed to identify and summarize the available evidence on the impact of anthropogenic point sources on the increase of ARG in the environment. Based on the authors' prior knowledge of the literature on this subject, the assumption was made that etiologic research on this review question would be uncommon, and that a narrowly focused question would not provide sufficient evidence to be meaningfully summarized. Thus, the review question was broadly formulated, permitting evaluation of a larger pool of evidence but also increasing the heterogeneity among the studies.
Most studies were considered to be unclear for risk of bias. The common reason for this was lack of information about potential confounders that might bias the observed relationship between proximity to a point source and levels of ARG in environmental media. The predominant confounder of concern to the review team was the introduction of antibiotics, ARB, or ARG from other sources that could differentially affect the

exposed and comparator sites. Many studies did not provide details about other possible contributors to resistance in the system or did not explain the location of other contributors and sampling sites. Studies with moderate to large spatial scales and no information about potential confounders were common. Most of these were considered to be an unclear or high risk of confounding bias.

We note that risk of bias assessment was conducted before the data extraction, which is a deviation from the original protocol. Though non-standard, this is unlikely to have introduced additional biases into the review findings because different reviewers evaluated the same study at the two different stages, and we extracted data from all studies including those considered at high risk of bias. The risk of bias assessment was conducted using subjective judgment, and despite reviewer consensus, this is a limitation of this review process.

As we noted, the most commonly evaluated point source was WWTP, which have been recognized to contain a large diversity of ARB and ARG (Rizzo et al., 2013). Human waste, which can include antibiotics, bacteria, and potentially ARB and ARG, is treated at WWTPs. However, ARG are still found after the treatment process, at the WWTP discharge, or at sites downstream from the WWTP (Rizzo et al., 2013), which is consistent with our findings, as most of the studies reported the highest levels of ARG (relative gene abundance or concentration) in river sites downstream from the point source (the WWTP) as compared to upstream sites or near the WWTP (compared to sites far from it). Only 5 studies assessing the impact of animal agriculture (3 terrestrial representing swine farms and a beef feedlot, and 2 in aquaculture representing fish farms) were included in the final pool of studies to review and reported mixed findings. The

small number of studies and the heterogeneity among the animal systems and gathered evidence, reveals insufficient scientific evidence about the impact of animal agriculture on a measurable increase of antibiotic resistance in the surrounding environment.

Overall, there was consistency in the results for the outcome data types (relative or absolute gene abundance or gene concentration) with most studies reporting a higher relative gene abundance and /or gene concentration downstream from the source (in unidirectional studies) or near the source (for those studies based on distance) across all source types.

Across all studies in the review, *sul1, sul2, tetA*, and intl1 were the most frequently detected ARG. The genes *sul1* and *sul2*, mainly found in Gram negative bacteria (Sköld, 2000), confer resistance to sulfonamide antibiotics, which are used in both animal and human practice, by modifying the dihydropteroate synthase related to protein synthesis. Among the large group of *tet* genes, *tetA* confers resistance to tetracycline via efflux pumps (Roberts, 2005). In the case of intl1, it codes for the integrase enzyme associated with many drug resistant bacteria (Mazel, 2006). Other ARG commonly detected were bla_{TEM}, $bla_{SHV,}$ and *ermB.* The first two confer resistance to β-lactam antibiotics (e.g. penicillins, cephalosporins, carbapenems) by encoding for β-lactamase enzymes, and *ermB* confers resistance to macrolide antibiotics through the modification of 23S by rRNA methylation (Szczepanowski et al., 2009).

Among those studies that conducted a statistical analysis, ANOVA or an equivalent non-parametric method was the most common approach. Such methods for comparison of means (unlike regression methods) cannot produce a quantitative summary effect measure when used to evaluate complex systems with a large number of relevant

comparison groups or covariates. A combination of regression methods such as the ones proposed by (Pruden et al., 2012) and (Amos et al., 2015) together with spatial analysis, as used in the study by (Czekalski et al., 2014) can provide a good framework to address some of the challenges related to bias, and quantification of the impact of point sources on the prevalence or concentration of ARG in the environment.

In light of this review process, the protocol (Williams-Nguyen et al., 2016) would have benefited from a few modifications (besides the ones we made *a posteriori*) to minimize the limitations and challenges encountered throughout the review. For instance, it would have been valuable to have an available tool to address the quality of the methodology and evidence provided by the studies for our specific review question; a possible solution to this would have been to include only those studies that explicitly defined a comparison of relevance to the review question or that conducted a statistical analysis for such a comparison.

Potential publication bias could not be assessed for this body of evidence. Publication bias is the exaggeration of treatment effect sizes caused by the propensity for journals to preferentially publish research showing statistically significant results (Song et al., 2013). Such bias can cause a meta-analysis to give a misleading picture of the effect size in question, such that the average effect size appears to exist when none is truly present or to exaggerate the magnitude of a significant effect size. Quantitative assessment of the presence of publications bias is possible when the distribution of sufficiently homogeneous effect measures can be examined via funnel plots and other methods (Duval et al., 2000). This review did not identify such a pool of quantitative results, thus publication bias could not be evaluated. Additionally, some existing evidence may not

have been identified by our search. Although some of the databases searched do index grey literature, our search strategy did not identify any. Furthermore, a meta-analysis was not conducted in this review for the same reason (lack of quantifiable homogeneous outcomes).

We identified a number of important considerations for future studies seeking to estimate the effect of a specific point source on environmental levels of ARG. Our review highlighted the need for epidemiological and/or ecological observational studies that control for selection bias, information bias, and confounding to the extent possible. Such studies will need to describe and adjust for confounders (especially due to other sources of antibiotics or resistant bacteria and/or genes). A good example of such approach is the study by (Pruden et al., 2012). Additionally, there remains a need for studies where the data analysis provides effect measures such as odds ratios or risk ratios (for studies with ARG prevalence as the outcome data type) or mean differences (for studies with ARG concentration as the outcome data type) to quantify the magnitude or strength of the effect of the exposure (i.e. the point source) on the outcome (i.e. the prevalence or concentration of ARG in the surrounding environment), accompanied by measures of variability. Pruden et al. (2012), despite creating relevant generalized linear regression models of the relationship of interest, did not provide parameter estimates from these models which would be needed to quantify the effect WWTPs had on ARG after accounting for other sources such as AFOs, and conversely, the effect of AFOs had on ARG accounting for WWTPs (Pruden et al., 2012).

Similarly, researchers should use statistical methods to infer the significance of the study findings and report these along with study results. The most appropriate statistical

model(s) will depend on specifics of the study design and on the outcome of interest. Enhanced collaborative work between microbiologists, ecologists, and other scientists to provide expertise where needed will aid in successful efforts to conduct etiologic research.

There is no doubt that the increase of antibiotic resistant bacteria is a global health crisis, and that there is a need to understand and intervene on the dissemination pathways. The role of the natural environment in the dynamics of antibiotic resistance is an area of great interest and concern (Allen et al., 2010a, Singer et al., 2006a). Research on the issue must use methodology able to contend with the inherent complexity of environmental systems subject to flux as well as the necessarily observational nature of most scientific evidence.

This systematic review provides a strong imperative to improve research methods in order to provide interpretable, quantitative information about the effect of point sources on resistance in the environment. Such information will ultimately be vital for developing effective interventions that will address resistance in the environment and benefit human and animal health.

Conclusions from both systematic reviews

Based on both systematic reviews, this is a summary of recommendations for future ARB and ARG environmental studies:

- Research question: Define question with PECO or other appropriate framework
- Study design:
    - Select adequate comparison groups
    - Maintain consistent sites for longitudinal studies
    - Sampling strategy/protocol

- Choose appropriate spatial scale for question and planned data analysis
- Consider temporal window

- Laboratory analysis
- Combination of molecular tools and culture-dependent methods

- Data analysis plan and reporting
- Provide detailed information about potential confounders
- Use analytical methods to control for bias when needed (e.g. regression analysis, spatial analysis)
- Report effect measures and measures of variability to quantify the effect of a source on levels of AMR in the environment/quantify the effect of a source on levels of AMR in the environment

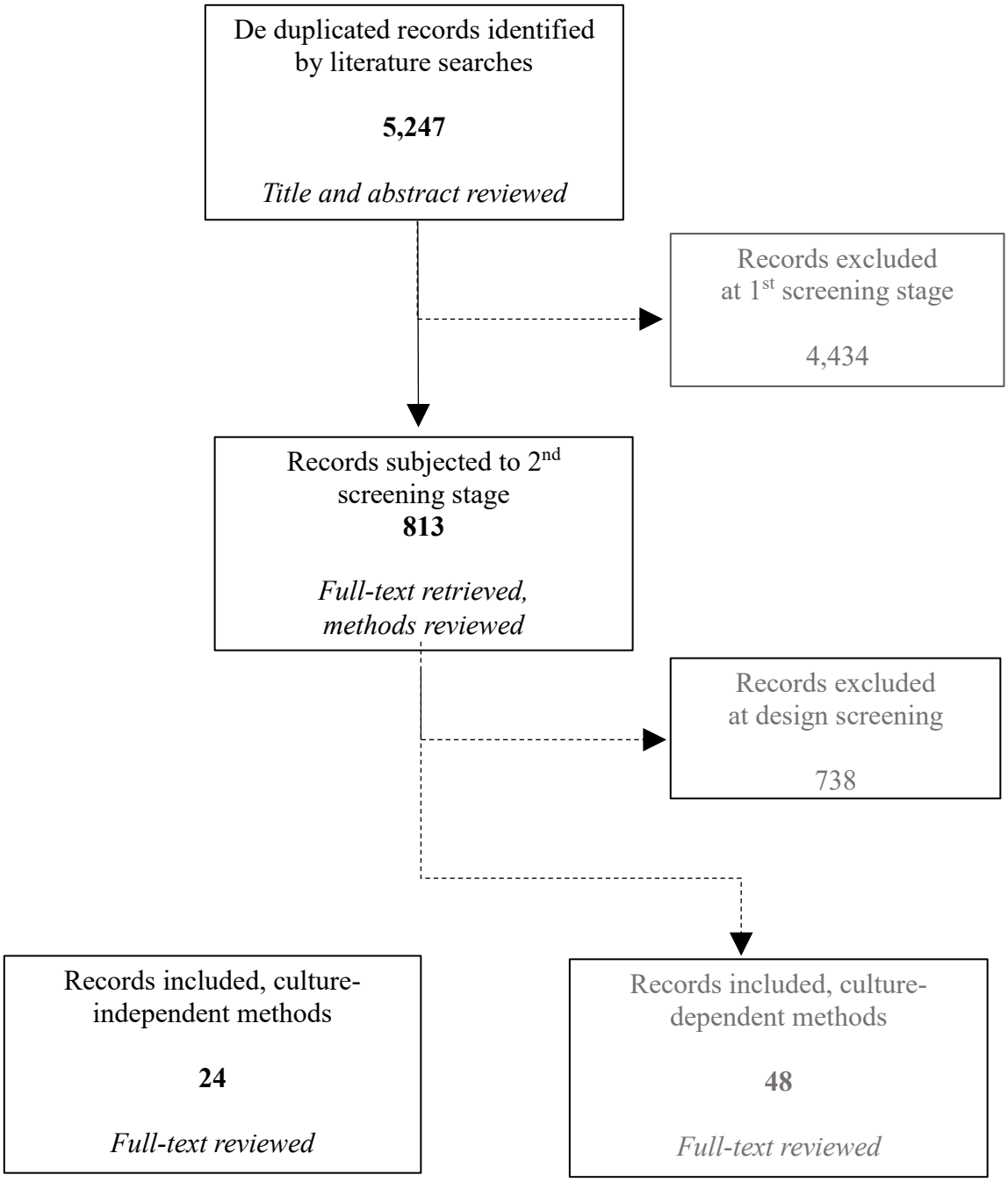


Figure 7. Flowchart summarizing the selection process for the studies for the ARG outcomes (the shaded boxes depict the articles excluded from the process and the records for the ARB outcome, not assessed in this chapter).

Table 2. Descriptive information for each of the 24 studies included in the systematic review assessing ARG outcomes. WWTP: Wastewater treatment plant.

Citation	Country/s	Spatial scale	Source type	Environmental media/System
Amos et al. (2015)	United Kingdom	50 km	Human waste (WWTP)	Sediment cores/ River
Berglund et al. (2015)	Sweden	3.5 km	Human waste (WWTP)	Sediment / River
Czekalski et al. (2012)	Switzerland	3.2 km	Human waste (WWTP)	Surface water, Sediment / Lake
Czekalski et al. (2014)	Switzerland	4 km	Human waste (WWTP)	Sediment / Lake
Harnisz et al. (2015)	Poland	400 m	Aquaculture (Fish farm)	Surface water / River
Hong et al. (2013)	United States	15 m	Terrestrial agriculture (Swine farm)	Ground water / River
Jia et al. (2014)	China	10 km	Terrestrial agriculture (Swine farm)	Surface water / River
Khan et al. (2013)	Pakistan	20 km	Human waste (City)	Sediment / River
Kristiansson et al. (2011)	India, Sweden	20 km	Human / industrial waste (WWTP receiving pharmaceutical manufacturing	Sediment / River

Citation	Country/s	Spatial scale	Source type	Environmental media/System
Lapara et al. (2011)	United States	8 km	Human waste (WWTP)	Surface water, Sediment / River, Lake
Lapara et al. (2015)	United States	> 960 km	Human waste (WWTP)	Surface water / River
Makowska et al. (2016)	Poland	Not reported	Human waste (WWTP)	Surface water / River
Marti et al. (2013)	Spain	200 m	Human waste (WWTP)	Sediment, Biofilm / River
Mceachran et al. (2015)	United States	10-20 m	Terrestrial agriculture (Beef feedlot)	Air
Pei et al. (2006)	United States	50 km	Human waste (City)	Sediment / River
Proia et al. (2016)	Spain	1.1 km	Human waste (WWTP)	Biofilm / River
Pruden et al. (2012)	United States	> 100 km	Human waste, CAFOs	Surface water / River
Rodriguez-Mozaz et al. (2015)	Spain	0.5 km	Human waste (WWTP)	Surface water / River
Sidrach-Cardona et al. (2014)	Spain	1.5 km	Human /industrial waste (WWTP, Antibiotic-production plant)	Sediment, Surface water / River
Stalder et al. (2014)	Not reported	5 km	Human waste (WWTP)	Surface water / River

Citation	Country/s	Spatial scale	Source type	Environmental media/System
Tamminen et al. (2011)	Finland, Sweden	1 km	Aquaculture (Fish farm)	Sediment / Sea
Uyaguari et al. (2011)	United States	100 km	Human waste (WWTP)	Surface water, Sediment / Sea
Xu et al. (2015)	China	Not reported	Human waste (WWTP)	Surface water / River
Zhang et al. (2013)	China	50 km	Human waste (City)	Surface water / River

Chapter 4. Antibiotic Resistance Genes in Freshwater Trout Farms in a Watershed in Chile

Introduction

Aquaculture production has risen globally over the past few decades, more than any other food production system. Between 1983 and 2013 it increased from 6.2 to 70.2 million tons at an average rate of 8.6% per year (FAO, 2018, Ottinger et al., 2016). As in any other farming system, the waste from aquaculture, which mainly includes fecal matter, nutrients, metabolites, and chemicals (Lawson, 2013), is released into the environment, but waste management varies depending on the location of the system, regulation requirements, and type of aquaculture production, among other factors.

Aquaculture production systems can be classified into four types based on the amount of infrastructure and management intervention humans provide: open, semi-closed, closed, and hybrid systems, although sometimes there is not a clear demarcation between them (Tidwell, 2012). Broadly, open systems consists of farming in enclosures, pens, or cages placed in natural bodies of water such as oceans, lakes, or rivers. Within open systems, there are different subtypes depending again on the level of human intervention. Waste removal in open systems generally occurs through natural processes, while in intensive open farms waste, especially solid waste, is also mechanically removed. Semi-closed systems, which consist of farming units such as ponds or raceways that divert water from natural sources such as a stream or river into the farm units, also rely upon natural processes to remove waste. In these systems, there is more control over the solid waste and it can be removed mechanically from the farm more easily. Closed systems consist of

intensively managed units where water is reused, environmental variables are controlled by the operator, and there is a physical barrier between the farm and the environment. In closed systems, water can be disinfected (unlike most open or semi-closed systems), and solid waste can be mechanically removed from the farm. Finally, hybrid systems are becoming more popular (e.g. hydroponics) as some of these combined systems may reuse or recycle their own waste within the system.

Chile is the eighth largest producer of aquaculture products globally, and the second after Norway in production of Atlantic salmon (*Salmo salar*) (Miranda et al., 2018). After Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*), coho salmon (*Oncorhynchus kisutch*), and blue mussels (*Mytilus chilensis*) are other commonly cultivated species in Chile (Miranda et al., 2018). For rainbow trout specifically, about 40,000 tons are produced annually (Subpesca, 2017).

Rainbow trout is often cultivated in freshwater semi-closed systems such as raceways (i.e. flow-through systems), and farms tend to be located in low-density farming areas (e.g. one farm per river). In raceways production, there must be a suitable source of water to provide sufficient volumes at the correct temperature year-round. Waste from these systems may be flushed into the receiving body of water, or processed on site.

As in any other production system, diseases affecting farmed animals (in this case fish), may arise despite management strategies to prevent them. In those cases, antibiotics are commonly used to control disease outbreaks (Wall et al., 2016). In aquaculture, antibiotics are usually provided as medicated feed (Austin et al., 2017). Despite the beneficial effects of antibiotics, they may also act as a selection pressure augmenting antimicrobial resistance (AMR) in the aquaculture environment (Watts et al., 2017).

In Chile, use of antibiotics in aquaculture is only allowed to be used for disease control, and not for disease prevention (Ministerio de Economia & Acuicultura, 2015). Six types of antibiotics are registered for its use in aquaculture in the country: amoxicillin, erythromycin, florfenicol, flumequine, oxytetracyline, and oxolinic acid (San Martin et al., 2014). Although data on antibiotic usage in aquaculture are not available for all the countries, larger quantities of antibiotics have been used in Chilean aquaculture compared to other countries like Norway or Scotland (Lyon, 2015, Lozano et al., 2018).

The effluent from fish farms may contain antibiotic residues, antibiotic resistant bacteria (ARB), and antibiotic resistance genes (ARG). Once in the aquatic environment, gene exchange can occur between bacteria, including environmental bacteria, and ARG can then be further disseminated through the natural environment (Baquero et al., 2008). Despite there being studies that have investigated ARG in aquaculture, there is a lack of quantitative studies attributing environmental ARG abundance to specific sources (Bueno et al., 2017, Williams-Nguyen et al., 2016). In order to characterize and manage the potential impact of these farms on the development and dissemination of ARG in the environment, being able to quantify their specific contribution is needed.

Although Chile has a relatively large aquaculture industry as measured by production, and in turn uses substantial amount of antibiotics, sparse data on AMR in aquaculture in Chile are available. Therefore, the objective of this study was to characterize the potential release and dissemination of ARG from freshwater trout farms into adjacent rivers in the Region de los Rios, southern Chile. It was hypothesized that, given the selection pressure of antibiotic use at these trout farms, ARG abundance would be higher downstream from

the trout farms compared to upstream sites. Furthermore, higher abundance of ARG encoding resistance to the antibiotics used at these farms was expected.

Materials and Methods

Study area

This study was conducted in rural areas within the Región de los Ríos, southern Chile. Five freshwater rainbow trout land-based raceway farms were enrolled in the study. Each one of the farms was located on a different river, but they were all part of the same watershed within this Region of Chile (**Fig 8**). All five farms belonged to a single company, and thus they had very similar management protocols. The general layout for the farms consisted of a flow-through system that used a continuously running source of water from the adjacent rivers. Specifically, water was diverted from the adjacent river into the farm's various raceways which contained the fish in different rearing stages. Then, the water exited all the rearing stages of the raceways and entered a common retention pond where solids settled out, and water then flowed back out to the same river. Waste from fish excrement and other organic and solid matter partially decanted at the bottom of each individual rearing stage raceway, and later it all settled out in common retention ponds at each farm. Retention ponds were emptied and cleaned once or twice per year, depending on the farm. The content from these ponds, which formed a thick sediment after being accumulated over time was removed, dried in the sun, and used as fertilizer in nearby agricultural fields (mostly for berry production). Between the retention ponds and the river there was a metallic screen preventing solid waste and fish from being released into the river. No other treatment (e.g. UV light) was applied to the effluent at these facilities, as it is not required in Chile.

Among the five farms, only one had a hatching stage. The rest of the production stages were common for the five farms, consisting of fingerling and growing phases (adults). Once the trout reached commercial weight (350-400 g) after 7-11 months, fish from all five farms were processed at the same processing plant. Of the total production, 90% was exported to other countries (mostly the U.S and Europe). In total, the five farms together produced approximately 2,000 gross tons of finished product per year.

Survey

A customized electronic questionnaire was sent to the company's management team. All the data and information provided by the company were collected under a confidentiality agreement. The questionnaire was written in Spanish and consisted of four sections: disease information, antibiotic use, other treatments, and management. Within each section, specific information for each of the five farms had to be completed. Follow-up clarifications to the survey were done via e-mail.

Sample collection

A longitudinal study was conducted, involving the repeated collection of samples around the five farms at four different time points (T1: March 2016, T2: October 2016, T3: November 2016, and T4: March 2017) except when environmental conditions prevented access. Composite river bed sediment samples, which consisted of multiple samples taken from a cross-section at each sampling site and deposited into a single tube (Falcon tubes), were collected at upstream (U1, U2, U3, with U1 being the furthest site from the farms) and downstream sites (D1, D2, D3, D4, D5, with D1 being the discharge site or a site immediately after the discharge site, and D5 being the furthest site downstream) from each farm at different distances from the farm (**Figs 9, 22**). In addition, samples from

retention ponds at each farm were collected (**Fig 23**). The top 2-3 cm of the river and retention pond bed sediment were collected manually using 50 mL Falcon tubes. Samples were taken at the upstream sites first, then at the retention ponds, and finally at the downstream sites, always following the same protocol. After collection, samples were immediately kept at 4°C until processing in the laboratory within 36 hours from collection.

A sample size calculation to determine the number of sampling locations per river was conducted assuming data was analyzed using a mixed effect model to account for repeated measures. Assuming normality, with 80% power, a confidence level of 95% , an effect size of a 3 log difference in ARG load between two groups (downstream and upstream), a standard deviation of 2 log ARG load (inferred from published literature with similar study design (Burch et al., 2013, Chen et al., 2013, Guo et al, 2013, Munir et al, 2011, Zhang et al., 2016, Zhuang et al., 2015)), and intraclass correlation coefficient (ICC) for within-river sites of 0.5, the minimum number of sites to sample was determined to be 7 per river (calculated using PASS 13 (NCSS, LLC. Kaysville, Utah, USA) (Vierron et al., 2007). Field sites were not selected randomly, but rather by convenience to target specific distances from each farm and to have safe access to the river. Information about specific sites was inferred from maps before visiting the sites and from knowledge provided by the trout company staff. The ESRI® App collector (ESRI) was used to collect Global Positioning System (GPS) coordinates at each sampling site.

Laboratory Methods

DNA extraction

DNA was isolated from all sediment samples (400 mg each) using the FastDNA® SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA), following manufacturer instructions with minor modifications: Step 4 (homogenization in the FastPrep® Instrument for 40 seconds at a speed setting of 6.0) was performed a total of 3 times, with 1 min incubation on ice between cycles; and in step 5 (centrifugation at 14,000 x g for 5-10 minutes to pellet debris), centrifugation time was 15 minutes. The final DNA elution volume was 100 µL and it was stored at -20°C until used for further analysis (microfluidic quantitative polymerase chain reaction technique or MF-qPCR).

Primer selection and validation

A total of 44 ARG were targeted for this study. Additionally, 16S rRNA and integrons (intl1, intl2, and intl3) were also included. The target genes were chosen based on a) being relevant to the study sites in Chile; and b) being representative of different molecular mechanisms of resistance, as well as ARG encoding for resistance to different antibiotic families. Primer sets and sequences for the gene standards of each one of the genes were either found in published literature (Sandberg et al., 2017) or designed for this study from all known gene allele sequences downloaded from GenBank® (Benson et al., 2008) and CARD (McArthur et al., 2013). Primer sets and standards were chosen based on universal gene specificity, similar annealing temperature at or near 60°C, amplicon size of less than 300 base pairs, and less than 60% GC content. The primer sets and standard sequences designed for this study were created from alignment of gene allele sequences using Basic Local Alignment Search Tool (BLAST, NCBI) with conserved sequence used as input into the Integrated DNA Technologies PrimerQuest Tool (IDT, Coralville, IA).

For all primer sets, the resulting amplicon of each gene was used as the standard sequence, including 20 base pairs beyond the primer annealing sites at both 5' and 3' ends of the amplicon. All primer sets and standard sequences were synthesized by IDT using the IDT gBlock® Gene Fragments technology to create the standard sequences. The 48 gBlock Gene Fragments standards were pooled and prepared into a dilution series, ranging from $2x10^6$ copies per µL to $2x10^0$ copies per µL, final concentration of each gene. The complete list of ARG, primers and sequences can be found in **Table 19**.

Specific Target Amplification

Before performing the MF-qPCR, it was necessary to pre-amplify the target genes in the sample DNA in a 14-cycle multiplex PCR called specific target amplification (STA), as recommended by Fluidigm®. The goal of the STA was to increase the copy number of the target genes to a detectable limit for amplification to occur on the microfluidic chip. The STA was conducted following previously published protocols (Ishii et al., 2013). Briefly, sample DNA and the gene standard dilution series were each pre-amplified to be able to retain original copy number per µL in the sample, using all target gene primers pooled at equal concentrations to amplify each gene equally. The primer pool was prepared by mixing reverse and forward primers for each gene target at final concentration of 0.2 µM of each primer. The STA master mix was prepared using 5 µL TaqMan™ PreAmp Master Mix (Applied Biosystems, Thermo Fisher Scientific™), 2.5 µL of the 0.2 µM primer pool, and 2.5 µL of sample or standard dilution DNA per reaction. The reaction was performed using a Veriti 96-well thermal cycler (Applied Biosystems, Thermo Fisher Scientific™) with the following conditions: 95°C for 10 min, followed by 14 cycles at 95°C for 15 s, and 60°C for 4 min. After the STA reaction, each

reaction was diluted 50-fold in qPCR grade sterile TE buffer (Invitrogen, Thermo Fisher Scientific™) and frozen at -20°C until used as the template for MF-qPCR. Results were assessed for biases as previously described (Ishii et al., 2013).

Microfluidic Quantitative PCR (MF-qPCR)

To simultaneously quantify the entire ARG array in this study, a Fluidigm® microfluidic quantitative PCR (MF-qPCR) was performed on two technical replicates of DNA from each sample. This is a high-throughput, highly sensitive method, that relies on a microfluidic platform to run a greater number of reactions at a time than in conventional 96 or 384-well qPCR. A more detailed explanation about this method can be found elsewhere (Ishii et al., 2013; Sandberg et al., 2017; Zhang et al., 2018), (Ahmed et al., 2018). Briefly, on one side of a Fluidigm 96.96 Dynamic Array™ (Fluidigm, South San Francisco, CA), 88 DNA samples, 1 No Template Control (NTC), and 7 standard dilutions were loaded (96 wells total) with 2.25 µL template, 2.5 µL 2X SsoFast™ EvaGreen® SuperMix with Low ROX (BioRad), and 0.25 µL 20X DNA Binding dye (Fluidigm®) in each well. The other side of the array contained the target gene assays, each run in duplicate (96 wells total), with 0.25 µL 20 µM combined forward and reverse primers, 2.5 µL 2X assay loading reagent (Fluidigm®), and 2.25 µL qPCR grade sterile TE buffer (Invitrogen, Thermo Fisher Scientific™) loaded into each well. The IFC Controller HX (Fluidigm®) was used to prime and load the array into the chip according to manufacturer's instructions. The Biomark HD system (Fluidigm) was used to conduct the real-time qPCR thermal cycling and record amplification in the chip, according to manufacturer's instruction for EvaGreen reagents. The thermal conditions were: thermal mixing at 70°C for 40 min and 60°C for 30 s, hot start at 95°C for 60 s, then 40 cycles of

96 °C for 5 s and 60 °C for 20 s, followed by melting curve analysis of 60 °C for 3 s, then slow heating to 95 °C at a rate of 1 °C/3 s.

Data Analysis

Fluidigm Real-Time PCR Analysis software version 4.1.3 was used to extract and analyze the raw data from the MF-qPCR under default settings with the quality threshold set to 0.65 and Ct threshold to 0.1ΔRN. Amplification and melting temperature curves for all the standard dilutions and NTC reactions for each gene were evaluated and reactions were manually failed if standard Ct values were > NTC. Then, using a customized Microsoft Access database (Microsoft® Office 2013), standard curves were generated for each gene using the original copy number before STA and the Ct values of the standard dilution reactions. The goodness-of-fit (adjusted R^2) and the amplification efficiency were calculated for each standard curve, resulting in R^2 of ≥ 0.90 and amplification efficiencies ranging from 90 to 110% for all genes. The linear regression equation from the standard curves was used to calculate copy number for all sample reactions from the Ct values. Technical replicates with a difference of at least 1 cycle (Ct) between them were excluded. Results from this step were generated with the Access database as $\log_{10}$ copies per µL of DNA and exported as a spreadsheet for further analysis.

The Limit of Detection (LOD) of the assay was 2 copies per µL of DNA. Back-calculation to copies per gram of sediment was calculated by multiplying the number of copies in each sample by the DNA elution volume from the DNA extraction (100 µL) and by dividing that result for the amount of sediment that was used for DNA extraction (0.4 g). Final quantitative data for each ARG were the arithmetic mean of the two technical replicates. “Non-detect data”, defined as those values that were below the limit

of detection, were handled using the following criteria: a) those ARG that presented non-detect data in > 95% of samples were excluded from the analysis, as that non-detect data was assumed to be 0 (not present); b) ARG that had non-detect data in more than 20% of the samples but less than 95% (>20%, <95%) and that had the majority of values ranging between 0-1 log copy per µL of DNA were replaced with 0 in the linear scale, as it was assumed those were truly non-detect data; c) ARG that had non-detect data in more than 20% but less than 95% of the samples (>20%, <95%) but the majority of values were >1 log copy per µL of DNA were replaced with ½ LOD (which was 0 in the log scale). Using ½ LOD was considered the least biased approach from a simulation study (unpublished data). Finally, d) ARG that had non-detect data in less than 20% of the samples (≤ 20%), ½ LOD was used to replace the non-detect data. A similar criteria approach has been used by other authors (Zhang et al., 2016).

Gene quantities were expressed in two ways: relative abundance (i.e. relative scale), the ratio between the copy number of each gene (in the linear scale) in a sample and the copy number of 16S rRNA in the same sample (Devarajan et al., 2015); and as absolute abundance (i.e. absolute scale), which was expressed as gene copies per gram of sediment sample. Before further statistical analysis, data were $\log_{10}$ transformed to meet normality assumptions. Then, using the gene data in the absolute scale (gene copies per gram of sediment) and with the goal of identifying clusters of ARG, a Principal Components Analysis (PCA) was undertaken using the function prcomp in R from the built-in R stats package, and the package factoextra (Kassambara & Mundt, 2017) for PCA visualization.

Multivariable analysis using linear mixed regression models (LMMs) were then conducted on a reduced ARG dataset that consisted of those ARG that had non-detects in

≤ 20% of the samples, and on those that had non-detected values in >20% and <95% of the samples, but the majority of values were >1 $\log_{10}$ copy per µL of DNA. The linear mixed regression models were fitted to the $\log_{10}$ transformed data using the lme4 (Bates et al., 2014) and the lmerTest packages (Kuznetsova et al., 2017) in R for linear mixed models (LMMs) with the function lmer (Bates et al., 2014, Kuznetsova et al., 2017). In the models, Farm (F1-F5) was considered a random effect, and Site (U1, U2, U3, RE, D1, D2, D3, D4, D5, and U1 as the reference level) and Time (T1-T4) as fixed effects (T1 as the reference level). The main comparisons of interest across sites were between U1 (furthest upstream site and reference level) and D1 (site either right at farm effluent discharge or closest downstream site from discharge), and between U1 and RE (retention pond) to assess differences between upstream/downstream and to assess the effect of the retention ponds on ARG abundance. Comparison between U1 and downstream sites (U1 and D1-D5) was also relevant to assess dissemination of ARG downstream from the farm.

Statistical significance was defined with an alpha level of 5%, and Satterthwaite's approximation was used to obtain the p-values for the F-test for each model, as suggested previously (Luke, 2017). Several models were then evaluated from less to increased complexity (from the most basic model with only random effects to the most complex model that included an interaction between the fixed effects). Model fit was conducted using the anova function from the stats package in R, and the decision to keep the most parsimonious model was guided by the AIC, BIC, and the p-value from the Chisq test. Model assumptions were checked through the inspection of residual plots following previously published recommendations (Winter, 2013). Estimated marginal means were

extracted from the LMMs using the emmeans package (Lenth, 2018), and magnitude changes between the sites and/or time points were expressed in $\log_{10}$. Plot visualization was conducted with ggplot2 (Wickham, 2016). All data analysis were done using R Studio (version 1.0.143 – © 2009-2016 RStudio, Inc (Team, 2017)), and Microsoft® Excel (2013).

Results

The trout company reported the use of two antibiotics on the farms: florfenicol and oxytetracycline. These antibiotics were provided as medicated feed in pellet form and were only prescribed by a veterinarian for treatment of disease outbreaks. The most commonly reported diseases at the farms were: flavobacteriosis in fingerlings (2-12 g weight range) which was treated with florfenicol, and 'strawberry' disease (disease of unknown etiology but thought to be caused by a *Rickettsia*-like organism (Christie et al., 2018, Lloyd et al., 2011, Metselaar et al., 2010) in adults between 200-300 g weight, treated with florfenicol and/or oxytetracycline. Historically, these farms have only used those two antibiotics. Antibiotic doses were reported as mg of antibiotic per kg of fish treated, or as grams of antibiotic used per biomass (ton of fish treated). More details about the specific diseases and treatment per farm can be found in **Table 3**. Among the farms, F2 had the highest antibiotic use on an annual basis, and F3 the lowest (**Fig 10**). These farms did not use any other type of treatment such as copper, aluminum sulfates, vitamins, or zinc, and they also did not use any vaccines or probiotics. To control diseases, additional management practices included: increasing salinity in individual raceways when there was a suspected disease outbreak, as it has been reported elsewhere to control *Flavobacterium columnare* (Suomalainen et al., 2005), removing individual

fish with gross lesions, and decreasing fish density per raceway. Products containing chlorine, such as sodium hypochlorite, were used on all farms for cleaning and disinfection purposes. Raceways were disinfected once per year with these products. The retention ponds were emptied either once or twice per year, depending on the farm, as described earlier.

The average distance from the most upstream site to the most downstream site across the five farms was 608.4 m (range: 326.4-951.1 m.) A total of 94 sediment samples were collected across all farms and time points (**Table 4**). For the data analysis, one sample was excluded due to laboratory assay failure, so the final dataset was comprised of 93 samples.

Out of all the ARG, ten were never detected in more than 95% of all samples (*bla$_{CMY}$, bla$_{VIM,}$ mecA, mecC, qnrA, vanA, vanB, vatB, vatC,* and *vatE*) and were thus excluded from further analysis. Eighteen ARG had non-detect data in 20-95% of the samples and the majority of the values were <1 log copy per µL of DNA (*aacA, aadA5, bla$_{KPC}$, bla$_{OXA}$, bla$_{CTX}$, bla$_{IMP}$, bla$_{NDM-1}$, bla$_{PER-2}$, bla$_{TEM}$, dfr13, ermB, mcr-1, qnrB, qnrS, sul3,* and *vgbB*). These ARG were not considered for further analysis as their quantities were too low to analyze. Eleven ARG had non-detected values in more than 20% of the samples (> 20%, < 95%) but the majority of values were >1 $\log_{10}$ copy per µL of DNA (*bla$_{SHV}$, ermF, floR, sul2, tetB, tetL, tetM, tetQ, tetS, tetW,* and *tetX*). Five ARG (including 16S rRNA) had non-detects in ≤ 20% of the samples: *qacG, strB, sul1, tetA,* and *tetC.*

The first three principal components (PC) from the PCA explained altogether more than 75% of the total variance and several ARG grouped according to the PCA. The ARG that

contributed the most to Principal Component 1 (PC1), which explained 47.9% of the total variance, were *strB, sul1, qacG, tetA*, and *tetC*; to PC2 (which explained 17.4% of the total variance) *tetQ, tetM,* and *tetL*; and to PC3 (which explained 12.6%) *tetS, tetL*, and *floR* (**Fig 11**). The loadings (or coordinates) for all the 16 ARG included in the PCA can be found in **Table 5**.

The most abundant genes across all farms at all time points expressed as average copies per gram of sediment (average copies per copy of 16S rRNA) were *sul1*, *tetA* and *tetC*. Abundance for *sul1* ranged from the lowest abundance at the middle upstream site (U2), being $2x10^{5}$ ($3.05x10^{-6}$) to the largest abundance at the retention pond (RE), which was $4.04x10^{8}$ ($4.08x10^{-2}$). For *tetA*, the sites with the lowest abundance were U2 and the upstream site closest to the farm (U3), with $2x10^{5}$ ($2.6x10^{-4}$), and the site with the largest abundance was RE, with $2.7x10^{8}$ ($2.27x10^{-2}$). The abundance for *tetC* ranged from the lowest abundance at the upstream site furthest from the farm (U1), being $2x10^{5}$ ($5.50x10^{-3}$) to the largest abundance at RE, being $3.05x10^{8}$ ($4.74x10^{-2}$).

The least abundant genes (from the 16 genes analyzed) across all farms and time points expressed as average copies per gram of sediment (average copies per copy of 16S rRNA) were *ermF, bla*$_{SHV}$, and *tetX*. For *ermF*, the abundance ranged from the lowest abundance at U3, with $2.50x10^{2}$ ($5.29x10^{-6}$) to the largest abundance at the discharge site (D1) with $6.4x10^{5}$ ($1.59x10^{-2}$). Abundance for *bla*$_{SHV}$ ranged from the lowest at the furthest site downstream (D5), with $3.29x10^{3}$ ($6.24x10^{-4}$) to the largest abundance at the downstream site following the discharge point (D2), with $1x10^{5}$ ($2.27x10^{-4}$). The abundance of *tetX* ranged from the lowest abundance at U3 and D5 with $1.40x10^{3}$ ($3.15x10^{-5}$) to the largest abundance at D1 which was $4.8x10^{6}$ ($7.01x10^{-5}$).

Overall, there was a statistically significant ($p<0.05$) effect of the site on ARG abundance for *qacG, strB, sul1,* and *tet* genes (*tetA, tetB, tetC, tetL, tetM, tetQ, tetS, tetW, tetX*). For these genes there was a 1.33 $\log_{10}$ magnitude increase on average at D1 compared to U1, and an increase of 1.54 $\log_{10}$ magnitude increase on average at RE compared to U1. Genes in which a statistically significant effect of the sampling site was not observed were bla_{SHV}, *ermF, floR,* and *sul2.* Nevertheless, the highest average abundance recorded for all genes except for *ermF, tetC,* and *tetX* was found in samples collected in the retention ponds (RE). This increase in gene abundance from U1 to D1 and from U1 to RE was expressed as the difference in estimated marginal means at these sites adjusted for sampling time and for the random effect of farm. For each gene, the estimated marginal means (EMM) and parameter estimates (95% CI) for these comparisons can be found in **Tables 6** and **7**. No statistically significant effect of sampling time (T1-T4) was observed for ARG abundance except for *ermF, tetM, tetQ,* for which higher abundance was observed at T4 compared to the other time points (1.38 $\log_{10}$ increase on average).

The comparison between U1 and downstream sites (D1-D5) yielded mixed results, since there was a statistically significant difference at different sites depending on the gene: for *qacG, strB, sul1,* and *tetA*, D2, D3, and D4 were significant; for *tetB* and *tetC* the difference was significant for all downstream sites (D2, D3, D4, D5); for *tetX* the difference was at D3 and D4, and for *tetW* only at D4. The analysis of the pattern of abundance revealed also mixed results: in the majority of the genes (*qacG, strB, tetA, tetX, tetW*) a higher abundance at D1 compared to D2 was observed, followed by a subsequent increase in the abundance in D3 and D4, and then another decrease at D5 (**Fig. 12**). In the case of *sul1* a similar pattern was observed, although the decrease at D2

was not present. For *tetB* the pattern was with higher abundance at D1 than at D2 followed by an increase at D3 and all the way up to D5. Finally, for *tetC*, there was an increase from D1 up to D5 (**Fig. 12**). Of all these genes, three of them presented an overall decrease in abundance from D1 to D5 (*tetA, tetX* and *tetW*) with a decrease on average of 0.90 $\log_{10}$. In contrast, for the remaining genes (*qacG*, *strB*, *sul1*, *tetB*, and *tetC*) there was an increase between D1 and D5 (1.07 $\log_{10}$ on average).

Discussion

The study presented herein investigated the role of freshwater trout farms in southern Chile as contributors to ARG release and dissemination into the natural environment. There is evidence that ARG can be found in the environment, but attribution to specific point sources, such as fish farms, is still lacking (Williams-Nguyen et al., 2016). Here study design was carefully considered since it is a critical factor to advance the knowledge on the environmental AMR field that needs improvement, as has been previously highlighted in a systematic review (Bueno et al., 2017).

Some of the details considered in the study design made here were: first, its longitudinal nature, since sampling the same field sites over time can help to account for temporal fluctuations. Second, the selection of a reduced spatial scale for the study sites for each farm (ranging from 326.4 to 951.1 m), that can help to decrease the influence of potential confounders, such as farms, wastewater treatment plants, and other aquaculture facilities which were present in the same watershed as the trout farms. Third, statistical analyses conducted in this study aimed at generating effect measures (mean difference of ARG abundance between sampling sites), which is lacking in the environmental AMR literature. However, all the concerns that were described in the systematic review

previously mentioned (Bueno et al., 2017) could not be addressed and some of the limitations of this study include low power (five farms, only a few sites upstream and downstream from each farm, and not the same number of sites per farm), limited temporal coverage (only four time points) and lack of measurement of physical-chemical and hydrological parameters. All these factors could potentially influence the outcome of the study (measurement of the ARG abundance) by either under estimating or over estimating it.

The hypothesis of a significantly higher ARG abundance downstream from the farms compared to upstream sites was met for 12 out of the 16 ARG modeled. Even when there was not a statistically significant difference, on average, ARG abundance was higher at downstream sites compared to upstream sites for all genes. Harnisz et al. (2015) (Harnisz et al., 2015) reported similar results for water samples collected upstream and downstream from a fish farm located in a river in Poland, finding a statistically significant increase of the abundance of genes downstream compared to upstream, as well as an increased diversity of *tet* genes.

A number of genes associated with resistance against the antibiotics used at the farms (oxytetracycline and florfenicol) were included in the ARG array. There were nine *tet* genes (*tetA, tetB, tetC, tetL, tetM, tetQ, tetS, tetW,* and *tetX*), which are associated with resistance to tetracyclines, including oxytetracycline, and all of these had significantly higher abundance downstream from the farms (among which *tetA* and *tetC* presented the highest abundance). The gene *floR*, which is a florfenicol resistance gene, had higher abundance downstream compared to upstream, but this difference was not statistically significant.

The gene *floR* has been found in other aquaculture settings including Chile, and has been found regardless of antibiotic use (Fernández-Alarcón et al., 2010, Watts et al., 2017). This gene clustered very closely in the PCA with *sul1*, *sul2*, *tetA*, and *tetW*. This grouping is similar to the ARG present in an *E. coli* plasmid recovered from a cow sample from Valdivia, Chile (Fernández-Alarcón et al., 2011). In another report, a conjugative IncA/C plasmid isolated from *Aeromonas salmonicida*, a fish pathogen, carried *floR*, *tetA*, *sul1*, and other ARG (McIntosh et al., 2008), which is also similar to what was found here. This could suggest a co-selection mechanism, with several ARG encoding resistance to different antibiotic classes, grouped together on the same plasmid (Baker-Austin et al., 2006).

The *tet* genes *tetA* and *tetC* are found in Gram negative bacteria and encode efflux pumps. Aquaculture settings have been suggested as environments with high diversity of *tet* genes, perhaps due to the wide use of tetracyclines (including oxytetracycline) to treat fish diseases (Roberts, 2012). The gene *tetA* in particular has been reported in high prevalence associated with fish pathogens such as *Aeromonas salmonicida* (Casas et al., 2005).These genes are often disseminated by plasmids (DePaola et al., 1995).

In addition to *tetA* and *tetC*, the other most abundant gene was *sul1*, which is also carried by plasmids, and it has been commonly found throughout aquatic systems (Zhang et al., 2009). The quantities for all these three genes (*sul1*, *tetA*, and *tetC*) ranged from 10^5 to 10^8 gene copies per gram of sediment (10^{-6} to 10^{-2} copies per 16S rRNA). Similar quantitative findings have been reported elsewhere. Abundance for *tetC* and *tetM*, and *tetA* and *tetH* was found to be augmented (10^7 and 10^4-10^5 copies per gram of sediment respectively) in samples collected at rainbow trout farm sites in the Baltic Sea compared

to samples collected more than 1 km away from the farm, where these genes were not detected (Tamminen et al., 2010). In water and sediment samples collected at freshwater aquaculture farms in China, the most abundant genes detected were *su1*, *sul2*, *sul3* (10^{-5} and 10^{-4} copies per 16S rRNA) and in lower levels *tetW, tetO, tetT*, and *tetM* were frequently detected (Gao et al., 2012). Similar results were obtained at another freshwater aquaculture study from China, where abundance of *tetM, tetO, tetX, tetS* and *tetW* genes ranged from $2x10^{-5}$ to $4.2x10^{-3}$ copies per 16S rRNA, and *sul1* abundance ranged from $3x10^{-4}$ to $1.1x10^{-2}$ copies per 16S rRNA (Xiong et al., 2015). These two studies only collected samples at the farm sites, so it is unclear if gene abundance was augmented given the lack of comparison group.

In coastal fish farms in South Korea, effluent water samples from multiple farms were collected. Among the targeted genes, *tetB* and *tetD* were the most prevalent, with relative abundance ranging from 10^{-2} to 10^{-2} copies per 16S rRNA. They also detected *sul1* at levels ranging 10^{-6} to 10^{-5} copies per 16S rRNA (Jang et al., 2018). Other studies from different aquaculture systems around the world have also reported common detection of *tet* and *sul* genes, but only qualitatively (e.g. (Akinbowale et al., 2007, Kim et al., 2004, Muziasari et al., 2016, Seyfried et al., 2010)).

Comparing these results with our study, genes that have been found throughout other aquaculture systems such as *tetM* or *tetS* were not detected in high abundance in our study. This could be due to geographic differences, type of aquaculture systems, or different field and laboratory methods used. Also, there may have been other genes or gene variants present but that were not detected in our study, perhaps because they were

not picked up by the MF-qPCR primers. In future studies, metagenomics analysis could be incorporated to look for other genes or gene variants.

Published quantitative studies of ARG abundance at aquaculture sites and their surrounding environment are sparse, with descriptive studies reporting presence/absence of genes being more common. In addition, there is not much consistency in the way ARG abundance is reported , as some studies report ARG abundance in the absolute scale (gene copies per gram of sample) and others in the relative scale (gene copies per 16S rRNA). It is thus relevant to report these results both ways so that results from different studies can be compared.

Normalizing to 16S rRNA (relative scale) has limitations because not all bacteria have the same number of copies of 16S rRNA, and in environmental samples there may be wide bacterial diversity. However, this has been the most common approach to report results from environmental samples to date (Czekalski et al., 2014, Selvam et al., 2012).

To get a better understanding of the resistome in relation to its taxonomic composition at aquaculture surrounding environments, a combination of culture dependent and culture independent high throughput methods should ideally be used, as it has been suggested for other food production systems (Noyes et al., 2016).

One of the limitations of high throughput methods (such as MF-qPCR) though, is the presence of non-detects (given small volumes of DNA and reagents and complex environmental samples). Different approaches have been used to handle this issue. Some authors have replaced non-detects with 0 (e.g.(Muziasari et al., 2016)), others with ½ limit of quantification (e.g.(Sandberg et al., 2017)), and others by imputing values (e.g.(Ahmed et al., 2018)). In the present study, non-detect data were treated following an

'in-house' criteria approach, somewhat similar to one previously used (Zhang et al., 2016), and non-detect data for those ARG kept in the final analysis were replaced with ½ detection limit, based on results from an in-house simulation study (unpublished data). This is still an area that needs further research to achieve standardization across studies so that biases can be minimized.

In the system studied in Chile, except for three out of the 16 genes analyzed, the highest ARG abundance across sites was found at the retention ponds. The solid waste from the farm's raceways was deposited in these ponds for periods ranging from six months to a year before it was cleaned out. Therefore, the accumulation of the farm's waste for long periods of time most likely created an ideal environment for ARG concentration. This finding is similar to other production systems such as swine or cattle where ARG have been detected in high levels in the waste lagoons, the equivalent of the retention ponds. For instance, a significantly higher *tet* gene abundance was found over time at lagoons that belonged to the operation with the highest antibiotic use among different cattle feeding operations (Peak et al., 2007). In another study assessing ARG from swine lagoons, it was shown that the highest concentration and diversity of ARG was at the waste lagoons compared to other sites (Koike et al., 2017).

After the retention ponds, the highest abundance of ARG across the five trout farms was at the downstream sites, significantly higher than the upstream locations depending on the gene and the specific downstream site. Despite this statistically significant difference, the biological significance of this increase is not clear and needs to be further evaluated. The increase of ARG abundance downstream indicated a potential for ARG dissemination from the trout farms into the surrounding environment. The relevance of

the average increase of 1.33 $\log_{10}$ remains however unclear. For three out of the nine genes that showed statistically significant differences across downstream sites, there was a decay from D1 to D5 of 0.90 $\log_{10,}$ which would take ARG levels to similar values as those found in U1 (upstream site). Also, this decay would indicate that after approximately 132.7 m (average distance from D1 to the furthest downstream site, D5), the effect of the farms on ARG abundance decreases. However, the majority of the genes (six out of nine) presented higher abundance at D5 compared to D1 of 1.07 $\log_{10}$, which would indicate a more likely downstream dissemination for these specific genes.

The potential for dissemination through plasmids, combined with the increased ARG abundance downstream from the farms should be considered, but results from this study can't be overstated, given the limitations about study design highlighted earlier. However, aquaculture farms should evaluate their waste management strategies and perhaps include monitoring of ARG long-term, especially as the industry keeps expanding.

Table 3. Summary of the most common diseases and treatment for each disease at the five farms (F1-F5).

Farm	Disease	Mortality (%)	Disease frequency	Treatment	Dosage (mg/kg)	Duration (days)	Age fish	Number raceways treated
F1	Flavobact*	4	Monthly	Florfenicol	20	14	Fingerlings	4
				Oxytet †	100	14		
	Strawberry	0.1	Monthly	Increased salinity	NI ‡	NI‡	Adults	4
F2	Flavobact	3	Monthly	Florfenicol	20	14	Fingerlings	6
	Strawberry	0.1	Montly	Florfenicol	20	14	Adults	6
				Oxytet	80	14		
F3	Flavobact	4	Biannual	Florfenicol	20	14	Fingerlings	2
F4	Flavobact	4	Monthly	Florfenicol	20	14	Fingerlings	2
				Oxytet	100	14		
F5	Flavobact	4	Monthly	Florfenicol	20	14	Fingerlings	4
				Oxytet	100	14		

* Flavobact: Flavobacteriosis

† Oxytet: Oxytetracycline

‡ NI: No information

Table 4. Sampling sites for each farm, for each time point, and distance from each one of the sites to the retention pond (RE) in meters (m). For sites, U indicates upstream and D downstream.

Farm	Site	Time	Distance (m) from each site to Retention pond (RE) in m
F1	U1	T1, T2, T3, T4	248.2
	U2	T1, T3, T4	193.6
	U3	T1	178.1
	RE	T3, T4	0
	D1	T1, T2, T3, T4	62.4
	D2	T1, T2, T3, T4	93.5
	D3	T1, T3, T4	120.5
	D4	T3, T4	185.7
Total distance from U1 to D4: 433.2 m			
F2	U1	T1, T3, T4	156.6
	RE	T1, T2, T3, T4	0
	D1	T1, T2, T3, T4	178.2
Total distance from U1 to D1: 326.4 m			
F3	U1	T1, T2, T3, T4	355.5
	U2	T1	313.4

Farm	Site	Time	Distance (m) from each site to Retention pond (RE) in m
	U3	T1	303.5
	RE	T1, T2, T4	0
	D1	T1, T2, T3, T4	134.8
	D2	T1, T2, T3, T4	261.9
	D3	T1, T2, T3, T4	336.8
	D4	T2, T3, T4	377.7
	D5	T2, T4	384.3
Total distance from U1 to D5: 767.6 m			
F4	U1	T1, T2, T3, T4	318
	U2	T1	310.1
	RE	T3, T4	0
	D1	T1, T2, T3, T4	109.7
	D2	T1, T2, T3, T4	153.4
	D3	T1, T3, T4	277.7
Total distance from U1 to D4: 564.1 m			
F5	U1	T1, T2, T3, T4	702.5
	U2	T1, T4	643.2

Farm	Site	Time	Distance (m) from each site to Retention pond (RE) in m
	U3	T1	588.1
	RE	T3, T4	0
	D1	T1, T2, T3, T4	177.3
	D2	T1, T2, T4	227.2
	D3	T2	297.5
Total distance from U1 to D3: 951.1			

Table 5. Loading values from the principal component analysis (PCA) for each one of the genes (ARG) for each principal component (PC). The percentage (%) is the amount of total variance explained by each PC (ARG are organized alphabetically).

ARG	PC1 (47.9%)	PC2 (17.4%)	PC3 (12.6%)
***bla*$_{SHV}$**	0.10	0.13	-0.01
ermF	0.40	0.22	0.81
floR	0.74	-0.55	**-0.25**
qacG	**0.93**	-0.09	-0.05
strB	**0.97**	0.02	0.12
sul1	**0.94**	-0.24	0.14
sul2	0.83	-0.47	-0.02
tetA	**0.89**	-0.31	0.19
tetB	0.81	0.28	-0.15
tetC	**0.85**	0.40	-0.04
tetL	0.26	**0.64**	**-0.48**
tetM	0.36	**0.66**	0.03
tetQ	0.44	**0.69**	-0.006
tetS	0.20	0.53	**-0.51**
tetW	0.84	-0.31	-0.24
tetX	0.44	0.28	0.79

Table 6. Estimated marginal means (EMM) with standard errors (SE) and parameter estimates for the comparison between D1 and U1. These mean differences are adjusted for sampling time and the random effect of farm. Mean differences are expressed in $\log_{10}$ gene copies.

Gene	EMM±SE		Parameter estimate (95% CI)
	D1	U1	
qacG	5.50±0.51	4±0.52	1.50 (0.74, 2.26)
strB	5.43±0.43	4.17±0.43	1.26 (0.51, 2.01)
sul1	5.86±0.45	4.58±0.45	1.28 (0.56, 1.99)
tetA	5.71±0.45	4.22±0.46	1.49 (0.69, 2.30)
tetB	4.37±0.42	2.99±0.42	1.38 (0.78, 1.99)
tetC	6.33±0.46	5.06±0.47	1.27 (0.50, 2.05)
tetL	3.60±0.30	2.73±0.30	0.87 (0.27, 1.48)
tetM	3.75±0.33	2.71±0.33	1.03 (0.28, 1.80)
tetS	3.67±0.28	2.57±0.29	1.10 (0.42, 1.80)
tetW	3.53±0.37	2.87±0.37	0.66 (0.06, 1.26)
tetX	3.82±0.26	2.86±0.27	0.96 (0.39, 1.53)

Table 7. Estimated marginal means (EMM) with standard errors (SE) and parameter estimates for the comparison between U1 and RE. These mean differences are adjusted for sampling time and the random effect of farm. Mean differences are expressed in $\log_{10}$ gene copies.

Gene	EMM±SE		Parameter estimate (95% CI)
	RE	U1	
qacG	6.31±0.57	4±0.52	2.30 (1.41 ,3.19)
strB	6.30±0.49	4.17±0.43	2.12 (1.25, 3.00)
sul1	6.73±0.50	4.58±0.45	2.14 (1.31, 2.97)
tetA	6.24±0.52	4.22±0.46	2.02 (1.08, 2.94)
tetB	5.24±0.45	2.99±0.42	2.25 (1.54, 2.96)
tetC	7.13±0.52	5.06±0.47	2.07 (1.16, 2.97)
tetL	4.16±0.35	2.73±0.30	1.42 (0.72, 2.12)
tetM	4.09±0.40	2.71±0.33	1.37 (0.47, 2.26)
tetS	4.36±0.35	2.57±0.29	1.79 (0.96, 2.62)
tetW	4.72±0.41	2.87±0.37	1.85 (1.15, 2.55)

Figure 8. Map of the Región de los Ríos, Chile, where the study took place, and the location of the farms.

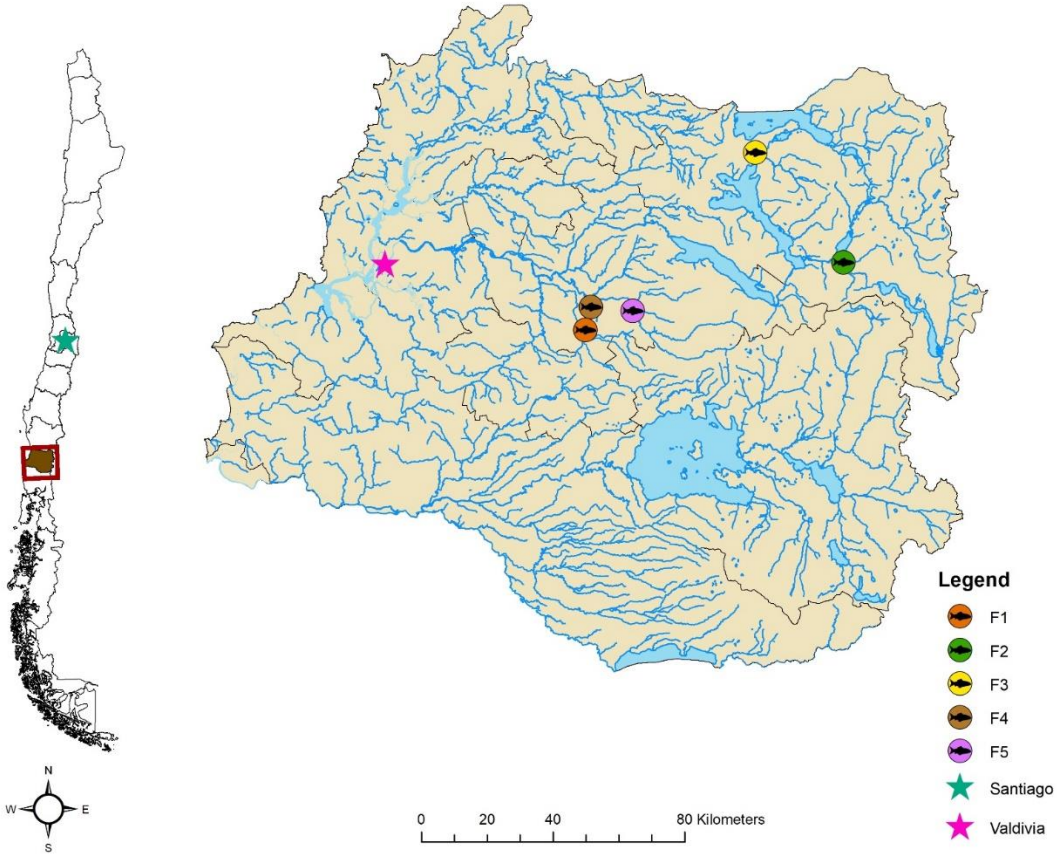


Figure 9. Depiction of the sampling sites upstream (U1, U2, U3), downstream (D1, D2, D3, D4, D5) and the retention pond site (RE) at a farm.

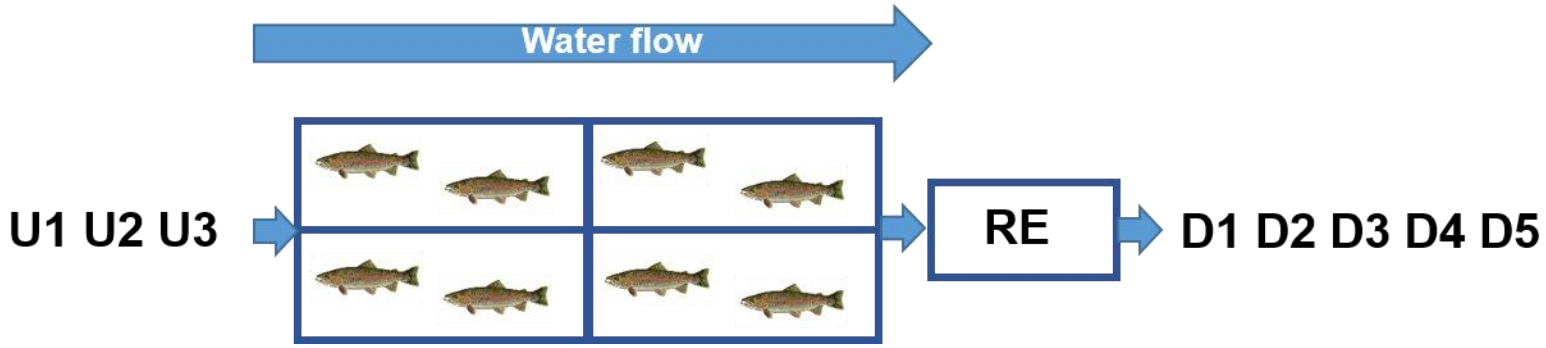


Figure 10. Annual antibiotic use for the two antibiotics used (florfenicol and oxytetracycline) at each one of the five farms.

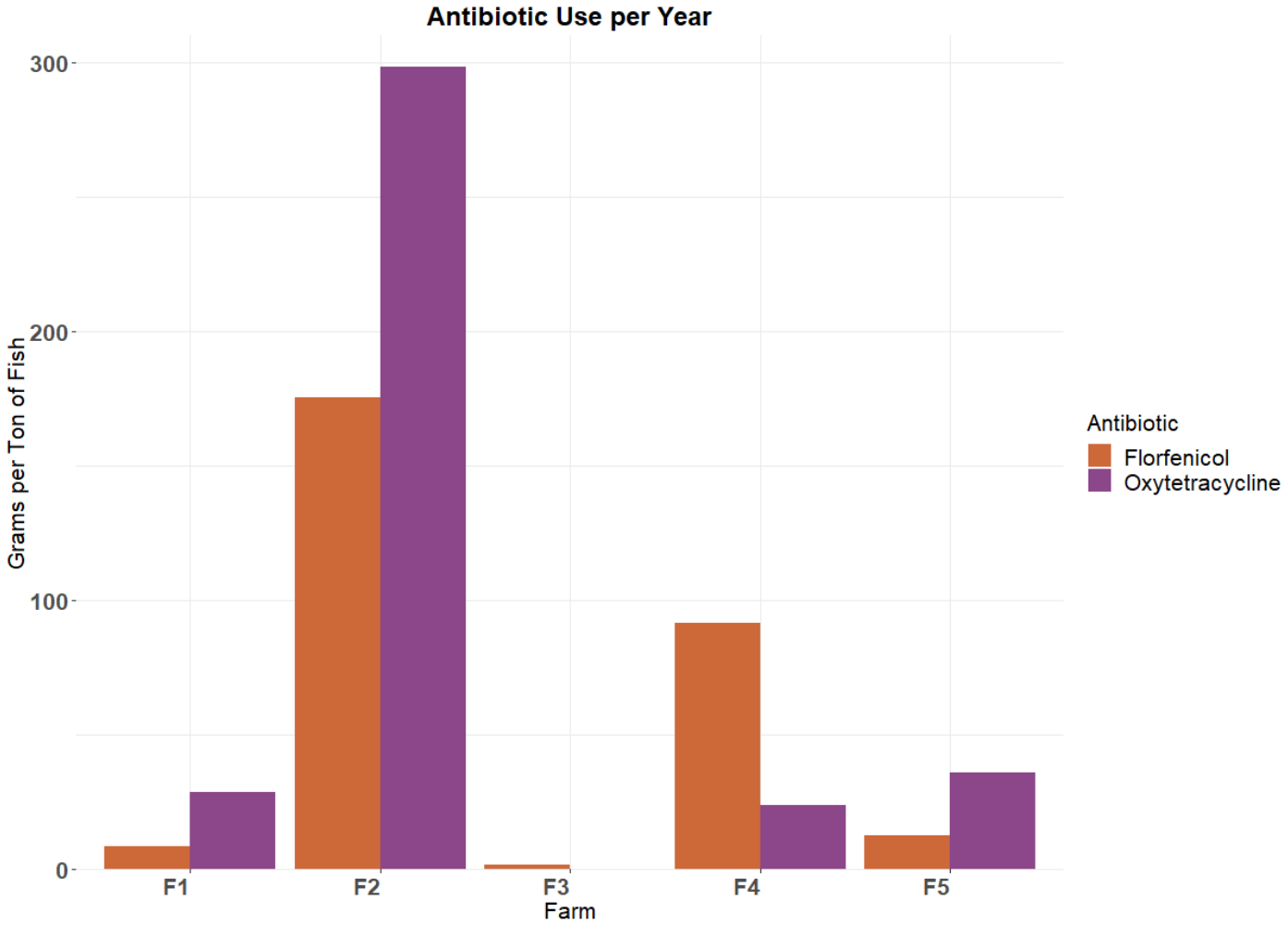


Figure 11. Variable plot from the principal component analysis (PCA) for all antibiotic resistance genes included.

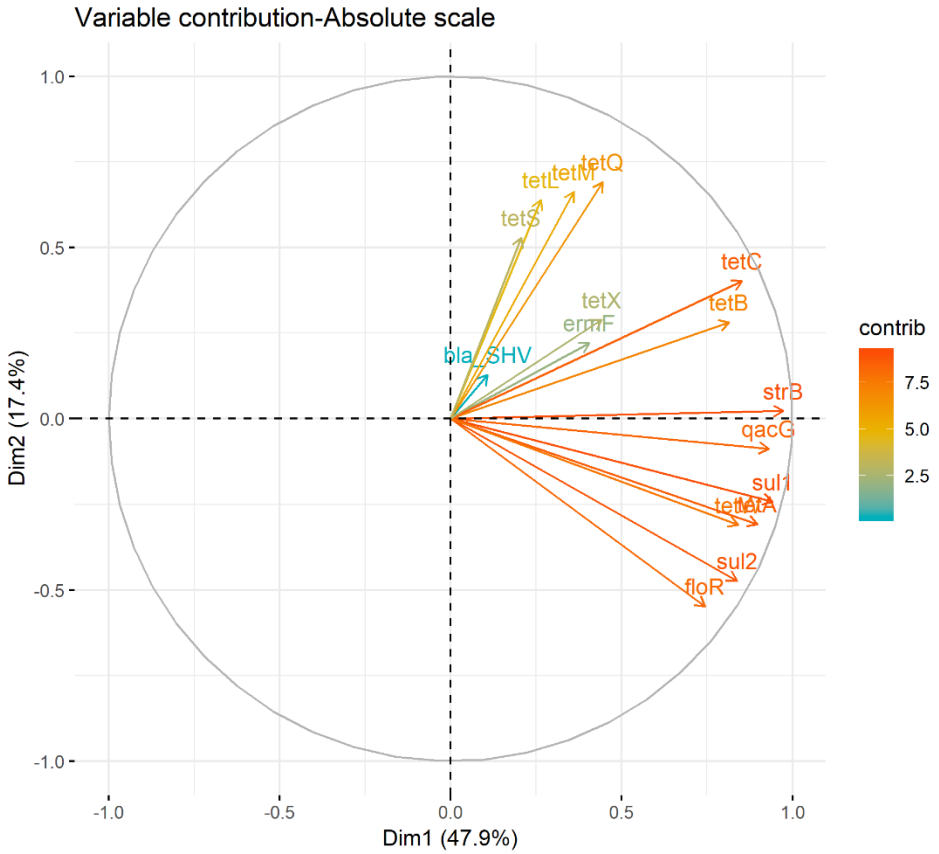
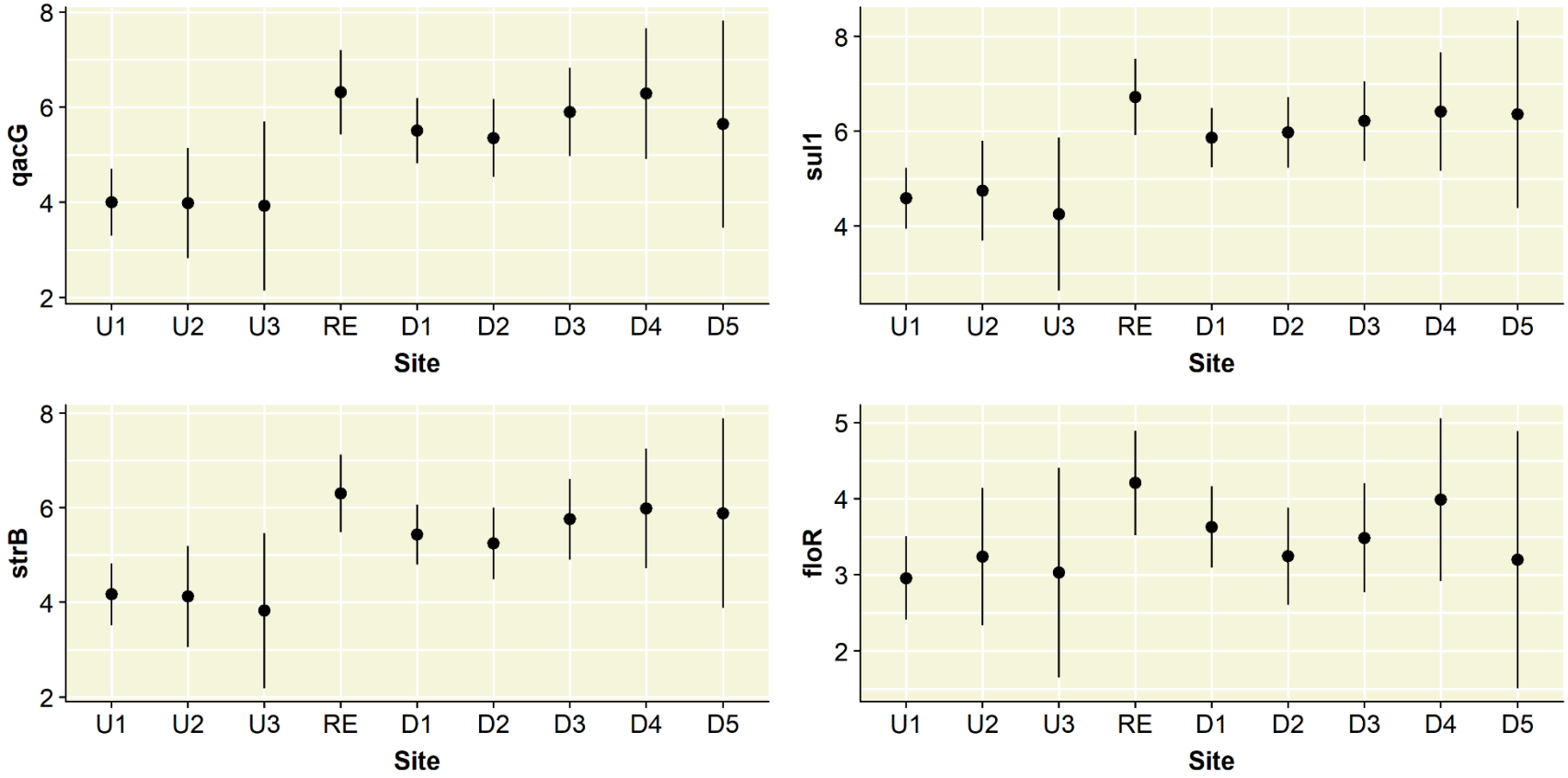
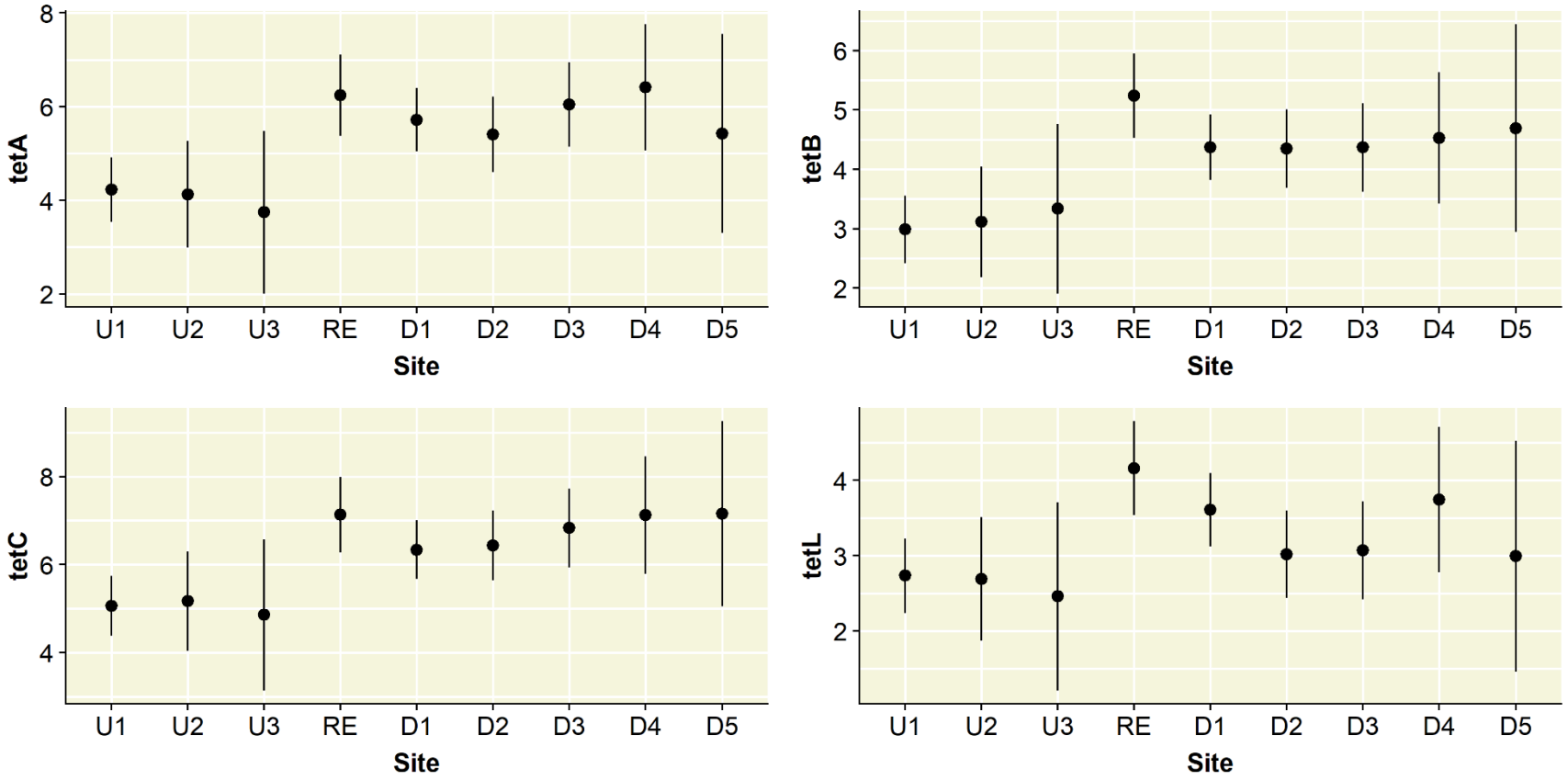
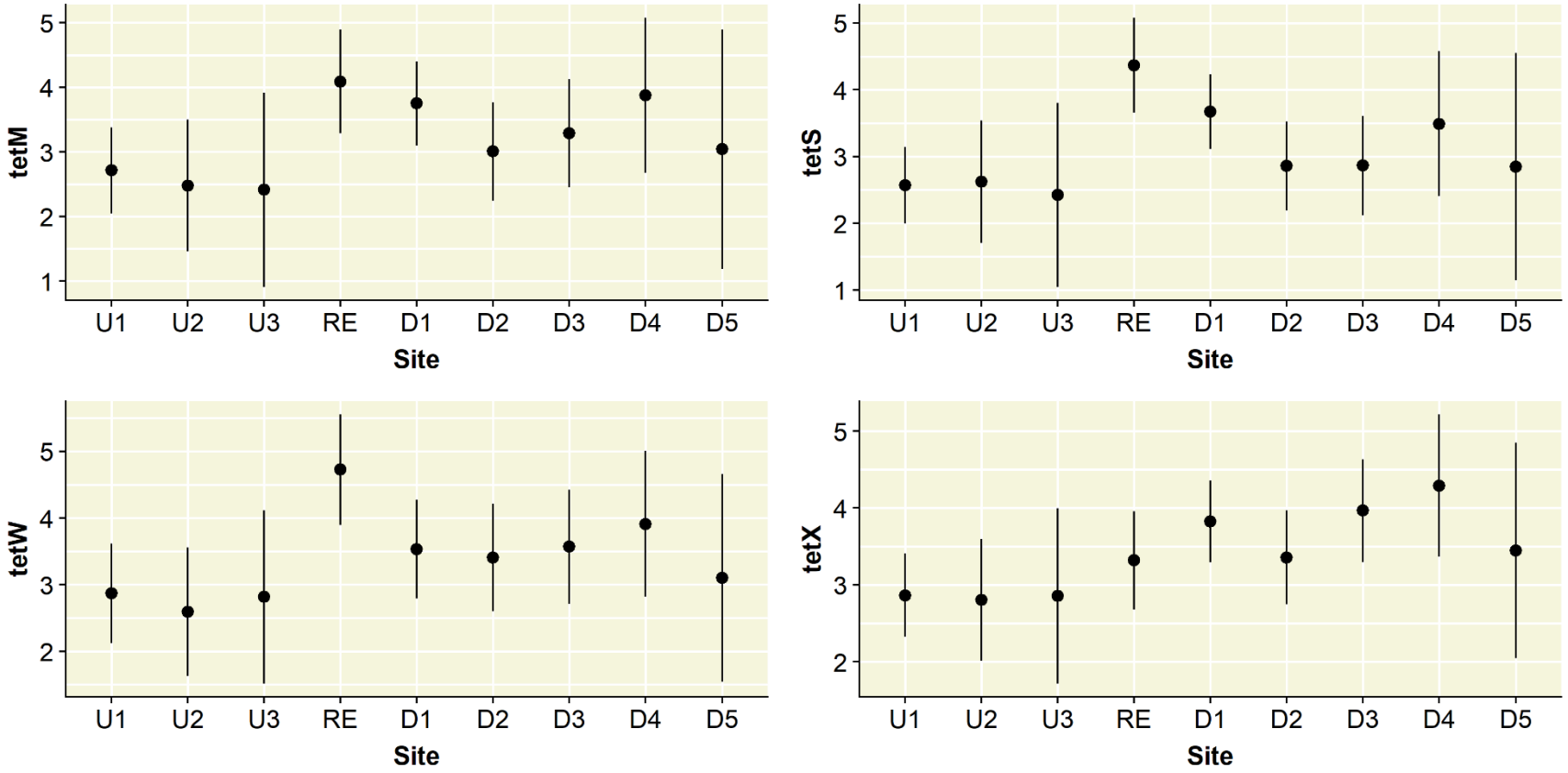


Figure 12. Estimated marginal means (and standard errors) for several of the genes analyzed with linear mixed models.




tetA
8
6
4
2
U1 U2 U3 RE D1 D2 D3 D4 D5
Site
tetB
6
5
4
3
2
U1 U2 U3 RE D1 D2 D3 D4 D5
Site
tetC
8
6
4
U1 U2 U3 RE D1 D2 D3 D4 D5
Site
tetL
4
3
2
U1 U2 U3 RE D1 D2 D3 D4 D5
Site


tetM
tetS
tetW
tetX
1
2
3
4
5
U1
U2
U3
RE
D1
D2
D3
D4
D5
Site

Chapter 5. Role of Wastewater Treatment Plants on Environmental Abundance of Antibiotic Resistance Genes in Chilean Rivers

Introduction

Despite the critical role of wastewater treatment plants (WWTPs) in treating and removing human waste prior to it being released into aquatic systems, they are also considered places that enhance antimicrobial resistance (AMR) development and spread (Berendonk et al., 2015, Karkman et al., 2017, Rizzo et al., 2013). Wastewater treatment plants receive the waste from hospitals, households, and industry and this waste may contain disinfectants, antibiotic resistant bacteria (ARB), antibiotic resistance genes (ARG), and unchanged antibiotics and/or antibiotic metabolites, given that humans only partially metabolize antibiotics (Grenni et al., 2018). All of these, in conjunction with the nutrient-rich environment at the WWTPs, makes it the perfect scenario to augment AMR. One of the main mechanisms by which AMR is enhanced at WWTPs is Horizontal Gene Transfer (HGT), when ARG are exchanged between bacteria of the same or different species, as well as between pathogenic and non-pathogenic bacteria (Martinez et al., 2017).

The effluent from a WWTP commonly discharges into rivers, lakes, and other surface waters, ultimately draining into watersheds. This effluent may contain ARG because WWTPs were not originally designed to remove them, but instead to reduce the microbial load and to remove the nutrient and solid contents (Lucas et al., 2014, Voolaid et al., 2017) and thus WWTPs are thought to play a role in the dissemination of ARG into the aquatic environment (Czekalski et al., 2014, Wellington et al., 2013, Woolhouse et al.,

2015, Voolaid et al., 2017). Another potential pathway of ARG dissemination from WWTPs is through wild birds. They have been observed feeding and drinking from aeration ponds (i.e. biological reactors) at WWTPs, which in turn may expose them to ARG present at WWTPs (Bean et al., 2018, Park et al., 2006). There is an increasing number of reports suggesting the role of wild birds as disseminators of AMR globally, especially given their ability to move over long distances (Ahlstrom et al., 2018, Carter et al., 2018, Smith et al., 2014, Vittecoq et al., 2016).

Despite considerable evidence of ARG dissemination from WWTPs, there is still a need to understand the attribution of environmental ARG to specific sources such as WWTPs in different ecosystems. Recent reviews have highlighted the need to improve study design, and to develop strong analytical methods to quantify the association between point sources like WWTPs and environmental levels of ARG (Bueno et al., 2017) (Williams-Nguyen et al., 2017). Furthermore, the human, animal, and ecosystem health consequences of ARG release and dissemination into the natural environment remains uncertain (Ashbolt et al., 2013).

To better understand some of these gaps, WWTPs in a watershed in Chile were evaluated. In South America, and Chile in particular, there are very few published studies assessing this issue. One study conducted in Antofagasta, northern Chile, found different phenotypic antibiotic resistant *Enterococcus spp* in two WWTPs and at the public hospital from this city (Silva et al., 2005). Another study, also conducted in WWTPs in the same city, evaluated the counts and proportions of resistant coliform bacteria both in the raw sewage and the treated effluent of the WWTPs. A high proportion of antibiotic resistant coliforms were found in the effluent, suggesting an environmental release of

antibiotic resistant bacteria from that WWTP (Silva et al., 2006). However, these studies evaluated ARB and not ARG, were conducted in a different region of the country, and they did not address dissemination because they did not collect samples at any river sites downstream from the plant. Also, they did not study the role of wild birds in disseminating ARB or ARG from the plant.

Therefore, the aims of this study were two-fold: a) to characterize and quantify the release and potential dissemination of ARG from three WWTPs into adjacent streams and rivers, all part of the same watershed in the Región de los Ríos, southern Chile. And b) to assess the role of wild birds as an indirect pathway of biological dissemination of ARG from one of the WWTPs from this study. It was hypothesized that a) there would be higher ARG abundance at downstream sites from the WWTPs compared to upstream sites, and that b) non-migratory birds (i.e. residents all year around next to the WWTP) would have higher ARG abundance compared to migratory birds.

Materials and Methods

Study site

This study involved three WWTPs in rural areas in the Región de los Ríos, southern Chile (South America; **Fig 13**). These WWTPs were all operated by a single private company. Despite small differences among the three WWTPs (**Table 8**), the core treatment process was the same. Chilean legislation has specific requirements for release levels of different contaminants and bacterial loads from WWTPs into the aquatic environment. For fecal coliform bacteria, the maximum limit permitted in the effluent before discharging into a river is 1000 MPN/mL. However, the legislation does not address AMR (D.S. 90, 2000).

The waste from these three WWTPs was treated following a common process. They received input waste continuously from the nearby households, hospitals, and other medical care facilities (e.g. clinics), and released a continuous output. The effluent of these three plants discharged into a stream (one of them) and rivers (the other two), all part of the same watershed in this Region. Briefly, the treatment process was divided into three phases or treatments (Essal, 2017):

- Pre-treatment and Phase 1: Mechanical separation (removal of solids, sand and fat). As waste entered the plant, it first went through a screen that removed large floating solids (e.g. toiletries, rags, sticks), and then it went through a finer screen that separated smaller solids from liquid waste. All the removed solids were deposited into containers that were later taken to a landfill. Then, a sweeper removed the fat and sand that had not been removed earlier. After that, the primary treatment took place. During primary treatment, floating materials and sediments that were not removed earlier were removed. It consisted of a primary physical decantation of the solids and flotation of smaller particles. The liquid waste was then directed into the next phase (phase 2).

- Phase 2: Biological process or activated sludge. At the WWTPs of the study there were two biological reactors (aeration ponds) per plant. In this phase, the liquid waste, which had already been through several filters, went into large open reactors. In these reactors, oxygen was pumped constantly at approximately 0.55 mg/L. In this mix, an aerobic reaction took place where bacteria degraded the organic matter, and it decanted at the bottom forming a sludge. The total volume for each one of these reactors was approximately 396 m^3, and they always kept a constant volume. These reactors removed approximately 40 m^3 of volume per day. After the aeration ponds there was a secondary

decantation, which was the final step from phase 2. This was similar to the primary physical decantation, in which left over organic and suspended matter was decanted and removed. The sludge was treated by adding a polymer and a centrifugation step to decrease the retained moisture, and then it was dried mechanically and stored for about a week. After that, it was utilized as fertilizer for agricultural fields in the area. This was another potential pathway of ARG dissemination but it was out of the scope of this study.

- Phase 3 (Tertiary treatment): the remaining water coming from secondary decantation was disinfected with UV radiation before discharging into the adjacent stream or river. There were eight UV lamps of 120 watts each that worked constantly, and were replaced with new ones every 12,000 hours. The general rule was to use 19 milliwatts /cm^2 for disinfection. Lamps were disinfected with chlorine, but chlorine was not used to treat the waste itself before discharge into the river. The plants worked continuously every day of the year. Besides the discharge pipe, there was a storm water pipe that was used to relieve additional water flow from the WWTPs like heavy rain in the winter, and sometimes even to release untreated waste in rare occasions.

Study design

We conducted a longitudinal study, sampling four times during the span of one year, specifically T1: March 2016, T2: October 2016, T3: November 2016, and T4: March 2017. These dates were chosen based on feasibility to access sampling sites and to cover two different seasons when hydrological conditions varied (October-November being Spring, and March being the end of the summer when it is much drier). We collected composite river bed sediment samples, which consisted of multiple samples taken from a cross-section at each sampling site and deposited into the same tube (Falcon tubes) at

upstream and downstream sites from each WWTP (W1, W2, W3) at different distances from the WWTP. Upstream sites were U1 and U2, with U2 being the site closest to the WWTP. Downstream sites were D1, D2, D3, D4, and D5, with D1 being WWTP discharge site, and the other sites located downstream at different distances from D1 (**Fig 14**). The top 2-3 cm of the river bed sediment was collected at each one of the sites manually, using 50 mL Falcon tubes. Samples were immediately stored at 4°C until processing in the laboratory within 36 hours from collection. The same process was repeated across the four time points.

A sample size calculation to determine the number of sampling locations per river was conducted assuming data was analyzed using a mixed effect model to account for repeated measures. Assuming normality, with 80% power, a confidence level of 95% (one-tailed), an effect size of a 3 log difference in ARG load between two groups (downstream and upstream), a standard deviation of 2 log ARG load (inferred from published literature with similar study design (Burch et al., 2013a, Chen et al., 2013, Guo et al., 2013, Munir et al., 2011, Zhang et al., 2016, Zhuang et al., 2015)), and intraclass correlation coefficient (ICC) for within-river sites of 0.5, the minimum number of sites to sample was determined to be 7 per river (calculated using PASS 13 (NCSS, LLC. Kaysville, Utah, USA) (Vierron et al., 2007). Field sites were not selected randomly, but rather by convenience to target specific distances from each WWTP and to be able to access the river sites. Information about specific sites was inferred from maps before visiting them and from previous studies in the same area (Singer et al, unpublished data). The ESRI® App collector was used to capture the Global Positioning System (GPS) coordinates at each sampling site. Details on the river field sites can be found in **Table 9**.

Wild birds were captured and sampled once a month for a period of 13 months (from April 2016 through April 2017) around one of the WWTPs (W3). Six mist nets (2.4x7 m) were placed around the W3 perimeter, in two sites: one site at 500 m from the W3 in the SE direction, where 4 nets were placed, and the other site in the same direction, at 700 m, closer to the discharge site of the WWTP, where 2 nets were placed. Nets were set up just before sunrise and monitored every 30 minutes for a period of 5 hours.

A brief physical exam was conducted for each bird that was captured. Standard morphometric measurements consisting of weight, wing chord length, 8^{th} primary length, tail length, head to tail length, metatarsus length, body fat, and muscle mass were taken. Then, a fecal sample was collected one of two ways: either by using a modified protocol by (Vázquez et al., 2010) which, briefly, consisted of inserting a micro hematocrit tube moist and filled with phosphate-buffered saline (PBS) solution into the bird's cloaca and retrieving the fecal material into a sterile Eppendorf tube; or alternatively, a direct fecal deposit was collected with a sterile swab after placing birds individually inside boxes covered with sterile papers for an approximated time of 10 minutes.

Once all measurements and samples were collected, an official metallic band was placed on the left metatarsus of each bird when feasible (some species could not be banded due to their small size, or they were banded on the right metatarsus if an injury or physical defect was present on the left leg) and they were set free. All bird handling was approved by the Servicio Agricola Ganadero (SAG) through the Universidad Austral permits (permit number: 2756/2015) and was done by the same team of people every month. The same day of the month that bird sampling took place, 1 L water samples were collected from each one of the two aeration ponds at W3 (**Fig 24**). This sampling was

done under SEREMI de Salud de Región de los Ríos permits and was conducted for most of the sampling months, except for when it was not feasible. All samples were stored at 4°C until processing in the laboratory.

Laboratory Methods

DNA extraction

DNA was isolated from all sediment and water samples (400 mg each) using the FastDNA® SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA), following manufacturer instructions with minor modifications: Step 4 (homogenization in the FastPrep® Instrument for 40 seconds at a speed setting of 6.0) was performed a total of 3 times, with 1 min incubation on ice between cycles; and in step 5 (centrifugation at 14,000 x g for 5-10 minutes to pellet debris), centrifugation time was 15 minutes. The final DNA elution volume was 100 µL and it was stored at -20°C until used for further analysis (MF-qPCR). For the bird samples (200 mg each), the QIAamp® Fast DNA Stool Mini Kit (Qiagen) was used to extract the DNA.

Primer selection and validation

A total of 44 ARG were targeted for this study. Additionally, 16S rRNA and integrons (intl1, intl2, and intl3) were also included. The target genes were chosen based on a) being relevant to the study sites in Chile; and b) being representative of different molecular mechanisms of resistance, as well as ARG encoding for resistance to different antibiotic families. Primer sets and sequences for the gene standards of each one of the genes were either found in published literature (Sandberg et al., 2017) or designed from all known gene allele sequences downloaded from GenBank® (Benson et al., 2008) and CARD (McArthur et al., 2013). Primer sets and standards were chosen based on universal

gene specificity, similar annealing temperature at or near 60°C, amplicon size of less than 300 base pairs, and less than 60% GC content. The primer sets and standard sequences designed for this study were created from alignment of gene allele sequences using Basic Local Alignment Search Tool (BLAST, NCBI) with conserved sequence used as input into the Integrated DNA Technologies PrimerQuest Tool (IDT, Coralville, IA).

For all primer sets, the resulting amplicon of each gene was used as the standard sequence, including 20 base pairs beyond the primer annealing sites at both 5' and 3' ends of the amplicon. All primer sets and standard sequences were synthesized by IDT using the IDT gBlock® Gene Fragments technology to create the standard sequences. The 48 gBlock Gene Fragments standards were pooled and prepared into a dilution series, ranging from $2x10^6$ copies per µL to $2x10^0$ copies per µL, final concentration of each gene. The complete list of ARG, primers and sequences can be found in **Table 19**.

Specific Target Amplification

Before performing the MF-qPCR, it was necessary to pre-amplify the target genes in the sample DNA in a 14-cycle multiplex PCR called specific target amplification (STA), as recommended by Fluidigm®. The goal of the STA was to increase the copy number of the target genes to a detectable limit for amplification to occur on the microfluidic chip. The STA was conducted following previously published protocols (Ishii et al., 2013). Briefly, sample DNA and the gene standard dilution series were each pre-amplified to be able to retain original copy number per µL in the sample, using all target gene primers pooled at equal concentrations to amplify each gene equally. The primer pool was prepared by mixing reverse and forward primers for each gene target at final concentration of 0.2 µM of each primer. The STA master mix was prepared using 5 µL

TaqMan™ PreAmp Master Mix (Applied Biosystems, Thermo Fisher Scientific™), 2.5 µL of the 0.2 µM primer pool, and 2.5 µL of sample or standard dilution DNA per reaction. The reaction was performed using a Veriti 96-well thermal cycler (Applied Biosystems, Thermo Fisher Scientific™) with the following conditions: 95°C for 10 min, followed by 14 cycles at 95°C for 15 s, and 60°C for 4 min. After the STA reaction, each reaction was diluted 50-fold in qPCR grade sterile TE buffer (Invitrogen, Thermo Fisher Scientific™) and frozen at -20°C until used as the template for MF-qPCR. Results were assessed for biases as previously described (Ishii et al., 2013).

Microfluidic Quantitative PCR (MF-qPCR)

To simultaneously quantify the entire ARG array in this study, a Fluidigm® microfluidic quantitative PCR (MF-qPCR) was performed on two technical replicates of DNA from each sample. This is a high-throughput, highly sensitive method, that relies on a microfluidic platform to run a greater number of reactions at a time than in conventional 96 or 384-well qPCR. A more detailed explanation about this method can be found elsewhere (Ishii et al., 2013, Sandberg et al., 2017, Zhang et al., 2018, Ahmed et al., 2018). Briefly, on one side of a Fluidigm 96.96 Dynamic Array™ (Fluidigm, South San Francisco, CA), 88 DNA samples, 1 No Template Control (NTC), and 7 standard dilutions were loaded (96 wells total) with 2.25 µL template, 2.5 µL 2X SsoFast™ EvaGreen® SuperMix with Low ROX (BioRad), and 0.25 µL 20X DNA Binding dye (Fluidigm®) in each well. The other side of the array contained the target gene assays, each run in duplicate (96 wells total), with 0.25 µL 20 µM combined forward and reverse primers, 2.5 µL 2X assay loading reagent (Fluidigm®), and 2.25 µL qPCR grade sterile TE buffer (Invitrogen, Thermo Fisher Scientific™) loaded into each well. The IFC

Controller HX (Fluidigm®) was used to prime and load the array into the chip according to manufacturer's instructions. The Biomark HD system (Fluidigm) was used to conduct the real-time qPCR thermal cycling and record amplification in the chip, according to manufacturer's instruction for EvaGreen reagents. The thermal conditions were: thermal mixing at 70°C for 40 min and 60°C for 30 s, hot start at 95°C for 60 s, then 40 cycles of 96 °C for 5 s and 60 °C for 20 s, followed by melting curve analysis of 60 °C for 3 s, then slow heating to 95 °C at a rate of 1 °C/3 s.

Data Analysis

Fluidigm Real-Time PCR Analysis software version 4.1.3 was used to extract and analyze the raw data from the MF-qPCR under default settings with the quality threshold set to 0.65 and Ct threshold to 0.1ΔRN. Amplification and melting temperature curves for all the standard dilutions and NTC reactions for each gene were evaluated and reactions were manually failed if standard Ct values were > NTC. Then, using a customized Microsoft Access database (Microsoft® Office 2013), standard curves were generated for each gene using the original copy number before STA and the Ct values of the standard dilution reactions. The goodness-of-fit (adjusted R^2) and the amplification efficiency were calculated for each standard curve, resulting in R^2 of ≥ 0.90 and amplification efficiencies ranging from 90 to 110% for all genes. The linear regression equation from the standard curves was used to calculate copy number for all sample reactions from the Ct values. Technical replicates with a difference of at least 1 cycle (Ct) between them were excluded. Results from this step were generated with the Access database as $\log_{10}$ copies per µL of DNA and exported as a spreadsheet for further analysis.

The Limit of Detection (LOD) of the assay was 2 copies per µL of DNA. Back-calculation to copies per gram of sediment was calculated by multiplying the number of copies in each sample by the DNA elution volume from the DNA extraction (100 µL) and by dividing that result for the amount of sediment/fecal sample that was used for DNA extraction (0.4 g and 0.2 g respectively). Final quantitative data for each ARG were the arithmetic mean of the two technical replicates. "Non-detect data", defined as those values that were below the limit of detection, were handled using the following criteria: a) those ARG that presented non-detect data in > 95% of samples were excluded from the analysis, as that non-detect data was assumed to be 0 (not present); b) ARG that had non-detect data in more than 20% of the samples but less than 95% (>20%, <95%) and that had the majority of values ranging between 0-1 log copy per µL of DNA were replaced with 0 in the linear scale, as it was assumed those were truly non-detect data; c) ARG that had non-detect data in more than 20% but less than 95% of the samples (>20%, <95%) but the majority of values were >1 log copy per µL of DNA were replaced with ½ LOD (which was 0 in the log scale). Using ½ LOD was considered the least biased approach from a simulation study (unpublished data). Finally, d) ARG that had non-detect data in less than 20% of the samples (≤ 20%), ½ LOD was used to replace the non-detect data. A similar criteria approach has been used by other authors (Zhang et al., 2016).

Gene quantities were expressed in two ways: relative abundance (i.e. relative scale), the ratio between the copy numbers of each gene (in the linear scale) in a sample and the copy numbers of 16S rRNA in the same sample (Devarajan et al., 2015); and as absolute abundance (i.e. absolute scale) which was expressed as gene copies per gram of sample (sediment, fecal, or water). Before further statistical analysis, data was $\log_{10}$ transformed

to meet normality assumptions. Then, using the gene data from the river sites upstream and downstream from the WWTPs in the absolute scale (gene copies per gram of sediment) and with the goal of identifying clusters of ARG, a Principal Components Analysis (PCA) was undertaken using the function prcomp in R from the built-in R stats package, and the package factoextra (Kassambara et al., 2017) for PCA visualization.

Multivariable analysis using linear mixed regression models (LMM) were conducted on a reduced ARG dataset. The linear mixed regression models were fitted to the $\log_{10}$ transformed data using the lme4 (Bates et al., 2014) and the lmerTest (Kuznetsova et al., 2017) packages in R for linear mixed models (LMMs) with the function lmer (Bates et al., 2014). Models were fitted to the $\log_{10}$ transformed absolute copy gene number per gram of sediment. In the models, WWTPs (W1-W3) was considered a random effect, and site (U1, being the reference level, U2, D2, D3, D4, D5, with) and Time (T1-T4) as fixed effects. Statistical significance was defined with an alpha level of 5%, and Satterthwaite's approximation was used to obtain the p-values for the F-test for each model, as suggested previously (Luke, 2017). The most parsimonious model was chosen based on a step-wise regression approach, guided by AIC, BIC, and the p-value from the Chisq test from the anova function from the stats package in R. Model assumptions were assessed through the inspection of residual plots following previous published recommendations (Winter, 2013). Estimated marginal means were extracted from the LMMs using the emmeans package (Lenth, 2018), and magnitude changes between the sites and/or time points were expressed in $\log_{10}$.

For the bird samples, descriptive statistics were calculated for the dataset, and included a description of the total samples per month, the bird species captured during the study

period, as well as the ARG detected. A Principal Components Analysis (PCA) was conducted to evaluate ARG clustering in the bird samples using the function prcomp in R from the built-in R stats package, and the package factoextra (Kassambara et al., 2017) for PCA visualization. Also, ARG abundance for the same individual birds captured more than once throughout the study period (i.e. recaptures) were compared with two-tailed paired sample t-test.

Bird species were then grouped as migratory and non-migratory, and the abundance of three beta-lactamase genes (*bla*$_{KPC}$, *bla*$_{SHV}$, and *bla*$_{TEM}$) which had been commonly detected in the bird dataset were compared between the two groups. First, they were compared with a repeated measures ANOVA fitted to the $\log_{10}$ transformed outcome (ARG abundance for each individual beta-lactamase gene) using the aov function from the car package in R (Fox et al., 2011). The ANOVA assumptions were checked by plotting the residuals and using the leveneTest from the car package to check the homogeneity of variance assumption (Fox et al., 2011). Tukey's Honest Significant Difference test (Tukey HSD) was used as a post-hoc test. A MANOVA using the library mvnormtest (Jarek, 2012) was conducted to assess all the three beta-lactamases genes at the same time.

ARG abundance for a reduced number of individual genes was compared between the bird samples and the water samples from the aeration ponds for the months when the samples overlapped (i.e. months for which both water samples and bird samples had been collected) with repeated measures ANOVA. In this case, birds were grouped as one single ensemble for each month without differentiating species. Additionally, linear discriminant analysis (LDA) was conducted using the lda function from the MASS

package in R (Venables et al., 2002) aiming at discriminating between sample types (water samples from W3, migratory and resident birds) on the basis of gene abundance measurements. Genes included for these analysis were *bla$_{CTX}$*, *bla$_{SHV}$*, *bla$_{TEM}$*, *sul1* and *sul2*. Data was $\log_{10}$ transformed to meet normality assumptions before LDA analysis. For all the statistical analysis, statistical significance was defined with an alpha level of 5%. Plot visualization was conducted with ggplot2 (Wickham, 2016). All data analysis were done using R Studio (version 1.0.143 – © 2009-2016 RStudio, Inc (Team, 2017)), and Microsoft® Excel (2013).

Results

The total extension of the field sampling from the most upstream site to the furthest downstream site for the three WWTPs enrolled in this study was, on average, 255.97 m (89.5-433.6). A total of 69 sediment samples were collected from the three WWTPs at four time points (**Table 9**). Before data analysis, one sample had to be removed from the dataset because of laboratory assay failure, so the final dataset was comprised of 68 samples.

Nine genes were removed from further analysis because more than 95% of the samples had non-detect data: *bla$_{VIM}$*, *mcr-1*, *mecA*, *mecC*, *qnrA*, *vanA*, *vatC*, *vatE*, and *vgbB*. Eleven ARG had non-detect data in more than 20% of the samples (>20 %, <95%) and the majority of their quantities were < 1 $\log_{10}$ per µL of DNA. Twenty-two ARG had non-detect data in more than 20% of the samples (> 20%, < 95%) but the majority of the values were > 1 $\log_{10}$ per µL of DNA. Two genes had non-detect data in ≤ 20% of the samples: *sul1*, and *tetC*. Genes kept for further analysis were inferred based on the descriptive data assessment as it is explained later on.

The most abundant genes across the three WWTPs at all time points, expressed as copies per gram of sediment (and the corresponding gene copies per copy of 16S rRNA for each gene) were *sul1, qnrS, sul2,* and *strB*. The abundance for *sul1* ranged from the lowest abundance at U2, being $5.2x10^{3}$ ($1.68x10^{-2}$) to the largest abundance at D1, being $2.33x10^{7}$ ($3.81x10^{0}$). Abundance for *qnrS* ranged from the lowest abundance at U2, being $2.72x10^{2}$ ($4.88x10^{-3}$) to the largest abundance at D5, which was $1.07x10^{8}$ ($2.76x10^{-1}$). For *sul2,* the site with the lowest abundance was U2, with $1.39x10^{3}$ ($5.23x10^{-3}$), and the site with the largest abundance was D1, with $1.93x10^{7}$ ($1.69x10^{-1}$). For *strB,* the lowest abundance was found at U2, which was $6.93x10^{2}$ ($4.95x10^{-3}$) and the highest abundance at D1, which was $7.80x10^{6}$ ($1.32x10^{1}$). For specific WWTPs, the most abundant genes at W1 were bla_{SHV}*, tetC,* and *sul1*, at W2*: qnrS, sul1* and *sul2*; and at W3: *sul1, sul2*, and *strB*.

The least abundant genes across the three WWTPs and time points (out of the 16 genes analyzed) were *vanB, floR,* and *tetQ*. For *vanB,* the abundance ranged from the lowest at D5 with $2.50x10^{2}$ ($4.10x10^{-6}$) to the largest abundance at D2 with $2.60x10^{3}$ ($8.00x10^{-3}$). Abundance for *floR* ranged from the lowest at U2 with $2.50x10^{2}$ ($4.87x10^{-3}$) to the largest abundance at D2 with $1.04x10^{4}$ ($8.10x10^{-3}$). The abundance of *tetQ* ranged from the lowest abundance at U2 with $2.65x10^{2}$ ($4.87x10^{-3}$) to the largest abundance at D1 which was $3.51x10^{4}$ ($1.07x10^{-1}$). For specific WWTPs, the least abundant genes at W1 were *aacA, tetS*, and *dfr13*; at W2 were *vanB, tetX*, and bla_{KPC}; and at W3 were *vanB, tetS*, and *floR*.

Principal Component Analysis (PCA) was conducted to investigate clusters of ARG in the river sediment samples. The first three PC explained altogether 67.4% of the total

variance. The ARG that contributed the most to Principal Component 1 (PC1), which explained 48.3% of the total variance, were *strB, tetA, dfr13, bla$_{TEM}$, aadA5, bla$_{OXA}$,* and *sul2*; to PC2 (which explained 11% of the total variance) were *vanB, floR, bla$_{SHV}$* and *tetQ* ; and to PC3 (which explained 8.1%) were *bla$_{KPC}$,* and *ermF* (**Fig 15**). The loadings (or coordinates) for all the ARG included in the PCA can be found in **Table 10**.

Linear mixed models were conducted on those ARG that had non-detect data in less than 20% of the samples (≤ 20%) and on the genes that had non-detect data in less than 95% but ≥20% across all samples, with a total of 24 genes analyzed: *aacA, aadA5, bla$_{KPC,}$ bla$_{SHV}$, bla$_{NPS,}$ bla$_{OXA,}$ bla$_{TEM,}$ dfr13, ermB, ermF, floR, qacG, qnrS, strB, sul1, sul2, tetA,* tetB, *tetQ, tetC, tetS, tetW, tetX,* and *vanB.* The main comparisons of interest across sites were between U1 (furthest upstream site and reference level) and D1 (site either right at the WWTP discharge point or closest downstream site from discharge), and between downstream sites (D1-D5) to assess dissemination of ARG downstream from the WWTPs. There was a statistically significant increase of 1.43 $\log_{10}$ on average at D1 compared to U1 for 17 out of the 24 genes total (*aacA, aadA5, bla$_{NPS}$, bla$_{OXA}$, bla$_{TEM}$, dfr13, ermF, qacG, qnrS, strB, sul1, sul2, tetA, tetB, tetQ, tetW, tetX*). Even though for *bla$_{KPC,}$ bla$_{SHV}$, ermB, floR, tetC, tetS,* and *vanB* there was not a statistical significant difference between sites, the pattern of lower gene abundance at upstream sites and higher abundance at downstream sites was the same (**Fig 16**). On average, the increase from D1 to D5 was of 1.28 $\log_{10}$. There was not a significant difference across time points (T1-T4). For each gene that was statistically significant, the estimated marginal means and parameter estimates can be found in **Table 11**.

A total of 204 birds were captured at the W3 sites throughout the study period, but the analysis was performed on 160 samples. The remaining 44 samples either belonged to birds that could not be sampled, or there were laboratory related issues. In the final dataset, each sample equaled one bird. The number of birds (i.e. samples) collected per month varied from the highest number in June 2016 (n=20) followed by May 2016 (n=19), to the lowest number in April 2017 (n=2) and October 2016 (n=4). (**Tables 12, 13**). The number of different bird species captured throughout the study period was 19. The most common ones were: a migratory species, the white-crested elaenia (*Elaenia albiceps*) representing 28.75% of the total, and a non-migratory species, the house wren (*Troglodytes aedon*), representing 19.34% of the total. Other common species, also non-migratory, were: the black-chinned siskin (*Spinus barbatus*) (16.86%); the grassland yellow-finch (*Sicalis luteola*) (9.36%), and the tufted tit (*Anairetes parulus*), 8.13%.

The genes bla_{OXA}, bla_{VIM}, bla_{CMY}, bla_{IMP}, *dfr13*, *ermB*, *mecA*, *mecC*, *mcr-1*, *qnrS*, *tetS*, *tetX*, *vanA*, *vanB*, *vatB*, *vatC*, *vatE*, and *vgbB* were not detected in > 95% of the bird samples. The genes *aadA5*, *ampC*, bla_{NPS}, bla_{NDM-1}, bla_{PER-2}, *ermF*, *floR*, *qacG*, *qnrA*, *qnrB*, *strB*, *sul3*, *tetB*, *tetC*, *tetL*, *tetM*, *tetQ*, and *tetW* were not detected in > 20% samples (> 20%, < 95%) and the majority of their values were <1 $\log_{10}$ copy per µL of DNA. All these ARG were excluded from any further analysis. Genes kept for the analysis were those that had non-detect data in ≤ 20% of the samples, which were bla_{SHV}, and *sul1*, and genes that had non-detect data in >20% of the samples (> 20%, < 95%), but the majority of their values were >1 $\log_{10}$ copies per µL of DNA (*aacA*, bla_{KPC}, bla_{CTX}, bla_{TEM}, and *sul2*).

Across all birds trapped throughout the entire study period, and for all the ARG included in the analysis (n=7), the mean gene copies per gram of fecal sample was: $4.15x10^{4}$ (0-$1.43x10^{7}$), which corresponded to $8.07x10^{-2}$ (0-$1.25x10^{1}$) gene copies per copies of 16S rRNA. The most abundant genes found across all months and across all bird species were: *bla*$_{KPC}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *sul1*, and *sul2* (**Figs 17, 18**). Principal Component Analysis (PCA) showed that 55.3% of the total variance was explained by PC1 and the genes that contributed the most to PC1 were *sul1*, *sul2*, and *tetA*; 16% of the variance was explained by PC2 and the contributing genes were *bla*$_{KPC}$, *bla*$_{SHV}$, and *bla*$_{CTX}$; PC3 explained 10.5% of the variance and *bla*$_{TEM}$ and *bla*$_{SHV}$ contributed the most to PC3 (**Fig 19**).

Beta-lactamase gene abundance (*bla*$_{KPC}$, *bla*$_{SHV}$, *bla*$_{TEM}$) was compared between the most common migratory species, *Elaenia albiceps*, and the most common non-migratory species, *Troglodytes aedon*. This comparison was made for the months when these two species overlapped (i.e. the months when the migratory species was present at the study site). Those months were: April, September, November, and December (2016), and January, February and March (2017). The repeated measures ANOVA and Tukey's post hoc test showed a statistically significant difference between the migratory and non-migratory species overall (p=0.0083) and for the months of April 2016 and January 2017 (p=0.02) for *bla*$_{SHV}$ copies per gram of fecal sample. There was a statistically significant difference (p=0.029) for *bla*$_{TEM}$ copies per gram of fecal sample between the months of December 2016 and September 2016, November 2016, January 2017, March 2017, and between February 2017- September 2016, and January 2017. For *bla*$_{KPC}$, there was an overall significant difference (p=0.0029) between the months of April 2016 and September 2016, November 2016, January 2017; between the months of September 2016

and December 2016, February 2017; between November 2016 and the moths of December 2016, and February 2017; between January 2017 and the months of December 2016, January 2017, and February 2017; and between February 2017 and March 2017. For these three beta-lactamase genes individually, none of the comparisons were significant in the relative scale (gene copies per 16S rRNA). MANOVA analysis showed a statistically significant difference in the absolute scale for month for bla_{KPC} and bla_{TEM} ($p=0.016$), and in the relative scale month was significant for bla_{KPC} ($p=0.00065$).

From the 160 birds considered in the analysis, four of them were recaptured (i.e. captured twice throughout the study period). Three of them were *Elaenia albiceps* (April 2016-November 2016; December 2016-February 2017; and December 2016-January 2017), and one of them was *Troglodytes aedon* (captured in June 2016 and March 2017). There was not statistically significant differences ($p>0.05$) between the ARG abundance (both absolute and relative scales) for the comparisons between the same individuals over time and no clear patterns were observed.

Water samples were collected for the months of June, July, and September 2016, and January, February, March and April 2017. Genes that were not detected in > 95% of the samples were: bla_{VIM}, bla_{NDM-1}, *mcr-1*, *mecA*, *mecC*, *qnrA*, *vanA*, *vanB*, *vatB*, *vatC*, *vatE*, and *vgbB*. These genes were removed from further analysis. The percentage of non-detect data varied among the remaining genes, but the majority of their values were > 1 $\log_{10}$ copy per µL of DNA, so they were described further.

Among the included ARG, the most abundant genes in the water samples across all months expressed as gene copies per gram of sample (gene copies per copies of 16S rRNA) were *sul2:* $5.74\text{x}10^{7}$ ($2.85\text{x}10^{-2}$), *sul1*: $5.51\text{x}10^{7}$ ($2.66\text{x}10^{-2}$), *strB*: $3.88\text{x}10^{7}$ ($1.94\text{x}10^{-2}$), and

ermF: $1.95x10^{7}$ ($9.63x10^{-3}$). The least abundant genes were bla_{KPC}: $1.61x10^{3}$ ($4.05x10^{-6}$), *sul3*: $2.04x10^{3}$ ($2.22x10^{-6}$), *tetL*: $2.19x10^{3}$ ($2.80x10^{-6}$) and bla_{PER-2}: $6.75x10^{3}$ ($3.49x10^{-5}$).

A more diverse profile of ARG was detected in water samples compared to bird samples (both migratory and non-migratory species together), so only the ARG that overlapped between them were compared with repeated measures ANOVA. These genes were: *aacA,* bla_{KPC}, bla_{SHV}, bla_{CTX}, bla_{TEM}, *sul1*, *sul2*, and *tetA*. Repeated measures ANOVA showed a statistically significant difference between types of samples (water and birds) for the genes *aacA* (p=0.017), bla_{TEM} (p= 0.03), *sul1* (p= 0.00079), *sul2* (p=0.000234), and *tetA* (p=0.003) for gene copies per gram of sample (absolute scale), and for *sul2* (p=0.03) for gene copies per copies of 16S rRNA (relative scale). Tukey HSD did not show any statistically significant differences. Linear discriminant analysis showed that water samples separated from migratory bird samples and from resident (i.e. non-migratory) bird samples along Linear Discriminant 1 (LD1) based on gene abundance. Bird samples were not as clearly discriminated from each other, but still presented a slight separation along LD2 (**Figs 20, 21**). Results for the model showed on both absolute and relative scales that *sul1* followed by *sul2* had the greatest influence in LD1, and *sul1* followed by bla_{SHV} had the greatest influence in LD2 (**Table 20**). Group means (average of each gene within each sample type group) indicated on both the absolute and the relative scales that *sul2* followed by *sul1* had a greater influence on water samples; bla_{SHV} and bla_{CTX} on migratory bird samples; and bla_{CTX} followed by bla_{SHV} for resident birds (**Table 21**).

Discussion

Three wastewater treatment plants in rural areas of southern Chile were assessed for their impact on ARG release and dissemination into the natural environment. Two pathways of

dissemination were considered: a direct pathway through the river systems, and an indirect pathway through wild birds. There is a large body of literature reporting on the role of a variety of point sources, including WWTPs, as contributors of ARG development and spread in different parts of the world and for different waste treatment types. However, attribution to specific sources is still lacking, mostly due to the complexity of study design and data analysis required to assess environmental AMR, as it was emphasized in a systematic review (Bueno et al., 2017) and in a book chapter that highlighted the main epidemiological concepts when designing studies assessing the impact of WWTPs on AMR dissemination (Williams-Nguyen et al., 2017).

On an attempt to improve study design, we used a longitudinal design to account for temporal fluctuations, a tight spatial scale upstream and downstream from WWTPs to decrease the influence of potential confounders such as farms or other WWTPs, and quantification of ARG through a culture-independent approach (as opposed to ARG presence or absence) in combination with data analysis that generated effect measures (mean ARG abundance difference between sampling sites). Still, all the concerns that were described in the systematic review previously mentioned (Bueno et al., 2017) could not be addressed in this study, and some of the limitations include: low power (three WWTPs, only a few sites upstream and downstream from each WWTP, and not always the same number of sites per WWTP), only four time points, and physical-chemical and hydrological parameters were not measured, which are factors that could potentially influence the outcome (ARG abundance).

We estimated sample size following best practices and approaches inferred from the ecological literature (Downes et al., 2002), but power analysis to estimate the number of

samples for environmental AMR studies presents unique limitations: lack of information regarding meaningful differences in ARG abundance between sites (for example upstream-downstream), and no previous baseline or pilot data at the same sites that can help determine variation between sampling units.

Also, high throughput molecular methods such as the one used here (MF-qPCR) can simultaneously quantify multiple ARG in environmental matrices using small reagent and sample volumes (Muurinen et al., 2017, Sandberg et al., 2017, An et al., 2018). However, given these methods have not been extensively used to quantify ARG yet, handling non-detects and analyzing the resulting data is still not well standardized. Also, the method used in this study did not distinguish between live or dead cells, or between pathogenic or non-pathogenic microorganisms. Bacterial communities were not assessed which made it harder to know what exact factors could be influencing increases of specific ARG (Lorenzo et al., 2018). Ideally, a combination of culture-dependent and culture-independent methods should be used to get a better understanding of the resistome in relation to its taxonomic composition in aquatic environments near WWTPs.

Findings from the first part of this study assessing ARG abundance in rivers adjacent to WWTPs revealed that these WWTPs increased ARG abundance (*strB, sul1, sul2*) downstream from the plants on average by 1.43 $\log_{10,}$ and that ARG abundance increased for most ARG at the furthest sampling point downstream on average by 1.28 $\log_{10.}$ It would have been expected to observe a decay on ARG abundance downstream; however the furthest site downstream (D5) was only at a distance of 84.4 m from D1, so it is likely that due to sites being close together, there might be small fluctuations in ARG abundance. Other reasons for this increase are an influence of confounders such as

effluent from other sources or physical-chemical parameters. However, the biological significance of the increase in ARG abundance downstream from the WWTPs is still unclear.

Comparing ARG abundance in aquatic systems in relation to point sources such as WWTPs across studies needs to consider their wide variation in watersheds and study methodology, including collection of different environmental matrices (e.g. sediment or water). Beyond this caveat, studies conducted in different parts of the world have reported similar findings to our study. To name a few, higher abundances for several ARG including *sul1* were found downstream from a WWTP in Canada (Freeman et al., 2018). There was a (1.46±0.29) log increase in river water samples for *sul1* in that study, which was of similar magnitude to the study herein. In a large watershed scale study, a significant correlation was found between *sul1* levels and upstream anthropogenic sources including WWTPs (Pruden et al., 2012).

Increased *sul1* abundance at levels of 10^4 copies per mL of water sample was also found at the effluent of a WWTP in Poland, as well as *sul2*, with levels of 10^3 copies per mL (Makowska et al., 2016). These and other genes (*bla*$_{TEM}$, *qnrS, tetO,* and *tetW*) were increased at downstream sites from two WWTPs and from other anthropogenic sources in a river in Belgium, with abundance ranging 10^4 to 10^6 copies per mL of water sample (Proia et al., 2018). Significantly higher abundance for *bla*$_{TEM}$, *ermB, qnrS, sul1,* and *tetW* were found downstream from a WWTP in Spain as well (Rodriguez-Mozaz et al., 2015).

However, not all studies have reported increased ARG abundance downstream from WWTPs, and instead have reported either a decrease in ARG abundance or no change. For example, (Laht et al., 2014) did not find an increase of ARG abundance at

downstream sites from WWTPs in Finland, and (Yang et al., 2014) reported lower abundance and diversity of ARG at the effluent of a WWTP in Hong Kong using a metagenomics approach, compared to the influent of the same WWTP. There are other examples as it has been extensively reviewed before (Karkman et al., 2017). Regional differences, which entail human population size, antibiotic usage, economic level, watershed characteristics, and type of wastewater treatment technology will influence findings of ARG abundance in the environment.

In Región de los Ríos, where this study took place, and throughout Chile, 3rd and 4th generation cephalosporins are commonly used. Abundance of beta-lactamase genes in the sediment samples was not as high as other genes, but a co-selection mechanism could be happening, when genes of different antibiotic classes are physically linked in plasmids (Baker-Austin et al., 2006). However, recent studies have suggested that detection of ARG downstream from point sources such as WWTPs could be in largely part due to fecal pollution and not entirely due to selection from antibiotic use at these sites and at the environment (Karkman et al., 2018).

When comparing water samples from W3 aeration ponds with bird samples, it was evident that water samples differed from bird samples. Within bird samples, there was also some discrimination between migratory and non-migratory samples, but this differentiation was not as evident. It was clear though that *sul1* and *sul2* were genes that influence water samples the most and beta-lactamase genes (bla_{CTX}, *and* bla_{SHV}) influenced bird samples the most.

Beta-lactamases genes (bla_{KPC}, bla_{TEM}, *and* bla_{SHV}) were quantified in the bird samples, with higher abundance in the migratory species (*Elaenia albiceps*) compared to the non-

migratory species (*Troglodytes aedon*). This is the opposite to what we had hypothesized, as non-migratory species (i.e. resident) were expected to be more exposed to W3 than the migratory ones; however, this can be explained by the larger number of potential ARG exposure pathways for the migratory species during its long migration. *Elaenia albiceps* is a long-distance migrant, going from the tropics to Chile mid-October, where it stays through March-April, and then it returns to tropical grounds to winter there (John, 2004).

It was not feasible to track these birds with GPS technology to understand their specific ARG exposure pathways, and the recapture number was low (only 4 individuals were recaptured during the 13 months the study lasted), which also might explain the lack of statistical significance when comparing the same individuals over time. What is interesting from the bird recapture information is that the migratory species seemed to have site fidelity for W3 surroundings, as three out of the four recaptures were migratory species (*Elaenia albiceps*) and returned to the same place after several months. Future studies should, when possible, increase the number of sampling events to favor the probability of recaptures, and better yet, to track bird movements remotely with GPS devices (Arnold et al., 2016, Knight et al., 2016).

Designing studies to evaluate dissemination of ARG from WWTPs should incorporate wild birds considering improved methodology as suggested above, as WWTPs have been recognized as important habitats for some avian species, and even more so, as natural habitat loss increases due to anthropogenic impacts and birds require other water sources to survive (Gough et al., 2003). Migratory birds specifically are relevant in this context, because as it was observed in this study and as it has been reported elsewhere (Ahlstrom et al., 2018, Bonnedahl et al., 2014, Carter et al., 2018, Wang et al., 2017, Wu et al.,

2018), they can disseminate AMR long distances. In fact, it has been experimentally shown that they might be able to carry resistant strains and even spread them to other birds for long enough periods of time equivalent to a long distance migration (Sandegren et al., 2018).

Despite the increase in reports about the role of wild birds as disseminators of AMR globally, studies specifically evaluating ARG abundance in wild birds in association with point sources such as WWTPs are lacking. Also, most of these studies focus on species such as gulls (e.g. (Morrison et al. 2014, Stedt et al., 2014) or raptors (Marrow et al., 2009, Molina-Lopez et al., 2011), or they report ARB (Carter et al., 2018), especially resistant *E. coli*, while studies of ARG in Passeriformes, the predominant group in our study, have been less commonly evaluated. This makes it challenging to compare ARG abundance levels found in the birds from our study to others, and this may serve as baseline for future studies. Besides public health concerns, another component that is also important to consider, and one for which there is not enough information, is the potential health effect to wild birds acquiring ARG and ARB through exposure to human waste (Williams-Nguyen et al., 2016).

There is plenty of evidence that WWTPs are places that can benefit ARG development and exchange, and that they can act as point sources of ARG dissemination (Manaia et al., 2018, Rizzo et al., 2013). The three WWTPs evaluated in southern Chile were very similar to each other, but results can't be extrapolated to all WWTPs throughout the country, because their conditions may vary, as it was mentioned before (e.g. population served, waste received).

Chile is making improvements to combat AMR from a public health perspective (e.g. National Plan against AMR; (Ministerio de Salud, 2017)), but the role of the environment and point sources such as WWTPs are not usually considered or are still under studied. More studies like this one assessing other WWTPs in Chile would be helpful to have a broader understanding of their role as point sources of ARG dissemination.

Table 8. Individual features for each one of the three WWTPs included in the study (W1, W2, and W3).

WWTP / Features	**W1**	**W2**	**W3**
Population served	3,500	30,000	18,000
Type of wastee treated	Hospitals, households, schools, no industrial waste	Hospitals, households, schools, industrial waste	Hospitals, households, schools, industrial waste
Type of treatment	Primary, Secondary and Tertiary (UV)	Primary, Secondary and Tertiary (UV)	Primary, Secondary and Tertiary (UV)
Volume treated / time	11.82 liters/second / per day approximately (peak around	40 L/second	15.8 L/second
Discharge volume	600 m3/day	2,500- 3,000 m3/day (variation between summer	1.300 m3
Distance from the WWTP to the discharge point (river)	294 m	564 m	360 m

Table 9. Details of the sampling sites for each one of the wastewater treatment plants (W1, W2, and W3), with the distance between the discharge site (D1) and the other sites.

WWTP / Month	Site	Time	Distance (m) from each site to WWTP discharge site
W1	U1	T1, T2, T3, T4	263.5
	U2	T1, T2, T3, T4	116.3
	D1	T1, T2, T3, T4	0
	D2	T2, T3, T4	43.4
	D3	T2, T3, T4	68.4
	D4	T2, T3	171.2
	Total distance from U1-D4: 433.6 m		
W2	U1	T1, T2, T3, T4	7.09
	D1	T1, T2, T3, T4	0
	D2	T1, T2, T3, T4	9.38
	D3	T1, T2, T3, T4	46.4
	D4	T1, T2, T3, T4	67
	D5	T1, T2, T3, T4	84.4

WWTP / Month	Site	Time	Distance (m) from each site to WWTP discharge site
Total distance from U1-D5: 89.5 m			
W3	U1	T1, T2, T3, T4	135.5
	U2	T1, T2, T3, T4	64.7
	D1	T1, T2, T3, T4	0
	D2	T1, T2, T3, T4	19.4
	D3	T1, T2, T3, T4	64.1
	D4	T1, T2, T3, T4	110
	Total distance from U1-D4: 244.8 m		

Table 10. Loading values from the principal component analysis (PCA) conducted for the sediment samples. Percentage (%) is amount of total variance explained by each PC. Each ARG that contributed the most to each PC are bolded.

ARG	PC1 (48.3%)	PC2 (11%)	PC3 (8.1%)
aaca	0.83	-0.09	0.25
aada5	**0.90**	-0.12	-0.25
bla$_{KPC}$	0.36	0.08	**0.67**
bla$_{NPS}$	0.74	-0.06	0.12
bla$_{OXA}$	**0.90**	-0.09	-0.27
bla$_{SHV}$	0.20	**0.66**	0.33
bla$_{TEM}$	**0.91**	-0.02	0.26
dfr13	**0.94**	-0.11	0.08
ermB	0.07	0.23	-0.31
ermF	0.68	0.04	**0.53**
floR	0.29	**0.85**	0.04
qacG	0.77	-0.03	0.03
qnrS	0.03	-0.05	-0.02
strB	**0.97**	-0.07	-0.08
sul1	0.85	-0.15	-0.20
sul2	**0.89**	-0.12	-0.25
tetA	**0.97**	0.01	-0.13
tetB	0.57	-0.14	0.11
tetC	0.79	-0.05	-0.22
tetQ	0.46	**0.59**	-0.21

ARG	PC1 (48.3%)	PC2 (11%)	PC3 (8.1%)
tetS	-0.01	0.19	-0.40
tetW	0.78	0.14	-0.36
tetX	0.65	-0.02	0.41
vanB	0.05	**0.92**	-0.06

Table 11. Estimated marginal means (EMM), standard errors (SE), and parameter estimates D1 and U1 comparison for genes that were statistically significant ($p<0.05$). Mean differences are adjusted for sampling time and random effect of WWTP.

Gene	EMM±SE		Parameter estimate (95% CI)
	D1	U1	
strB	4.74±0.75	3.09±0.75	1.55 (0.61, 2.50)
sul1	5.24±0.71	3.65±0.71	1.59 (0.58, 2.60)
sul2	4.60±0.77	3.10±0.77	1.50 (0.44, 2.57)

Table 12. Species of birds captured during the study period and number of individuals per species (organized alphabetically).

Species	**Number of Birds**
Anairetes parulus	13
Aphrastura spinicauda	1
Cinclodes patagonicus chilensis	1
Cistothorus platensis	1
Colorhamphus parvirostris	1
Elaenia albiceps	46
Eugralla paradoxa	1
Glaucidium nanum	2
Leptasthenura aegithaloides	2
Phrygillus patagonicus	7
Phytotoma rara	4
Sephanoides sephaniodes	1
Sicalis luteola	15
Spinus barbata	27
Sylviorthorhynchus desmursii	2
Tachuris rubrigastra	1
Troglodytes aedon	30
Turdus falcklandii	2
Xolmis pyrope	1
Zonotrichia capensis	2
Total	**160**

Table 13. Number of birds captured per month.

Month	**Number of birds**
April 2016	15
May 2016	19
June 2016	20
July 2016	10
August 2016	4
September 2016	12
October 2016	4
November 2016	15
December 2016	15
January 2017	13
February 2017	18
March 2017	13
April 2017	2
Total	**160**

Table 14. Loading values from the principal component analysis (PCA) for the bird samples. The percentage (%) is the amount of total variance explained by each PC (ARG are organized alphabetically).

ARG	PC1 (48.3%)	PC2 (11%)	PC3 (8.1%)
aacA	0.80	-0.14	-0.12
aada5	0.82	-0.17	-0.04
bla$_{KPC}$	0.13	0.89	0.05
bla$_{SHV}$	0.10	0.62	-0.32
bla$_{TEM}$	0.27	0.14	0.90
sul1	0.98	-0.04	-0.01
sul2	0.96	-0.12	-0.03
tetA	0.97	-0.14	-0.02

Figure 13. Map of the Región de los Ríos with the WWTPs that were included in the study.

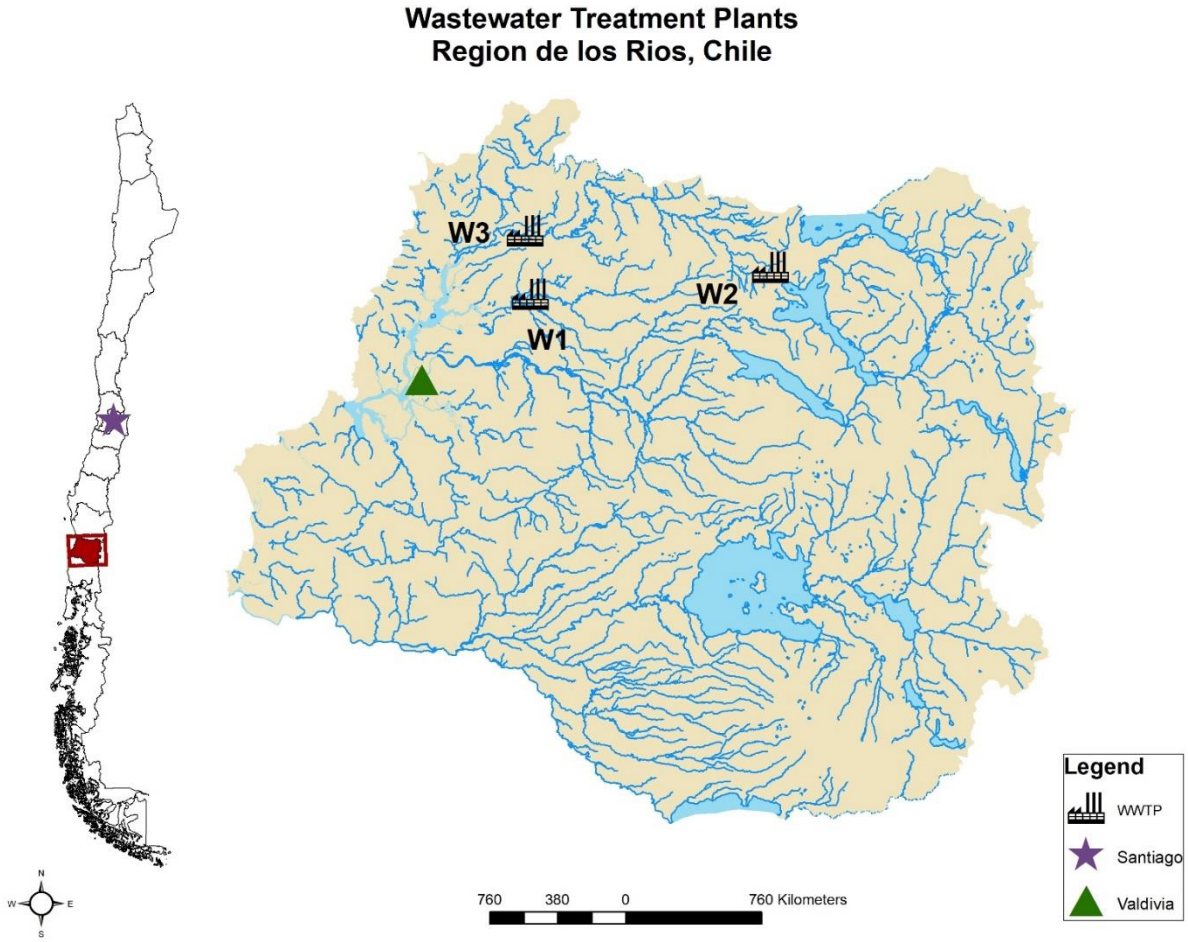


Figure 14. Depiction of the sampling sites upstream (U1, U2, U3), downstream (D1, D2, D3, D4, D5) from a farm and the retention pond site (RE).

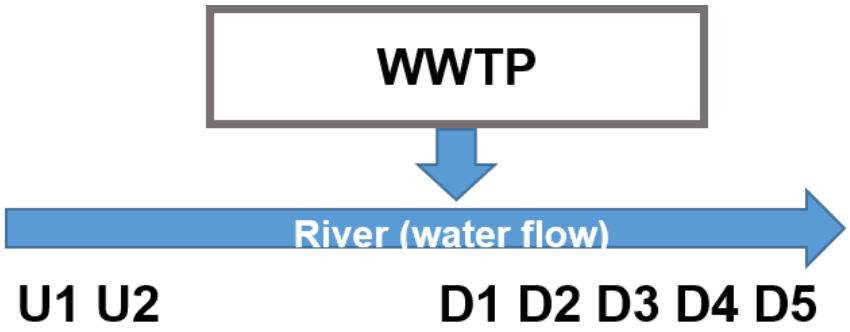


Figure 15. Variable plot from the Principal Component Analysis (PCA) for the WWTPs sediment samples.

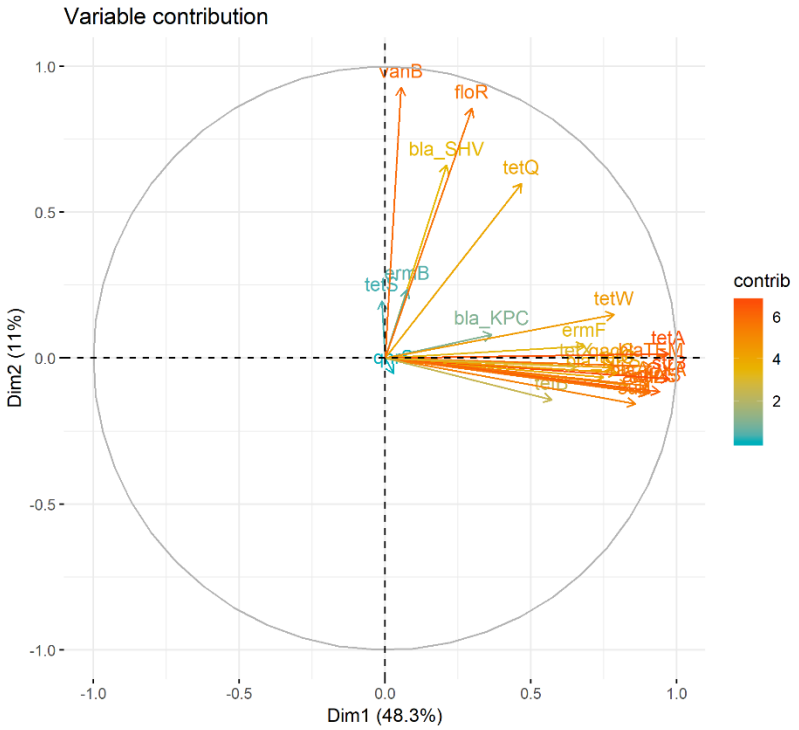
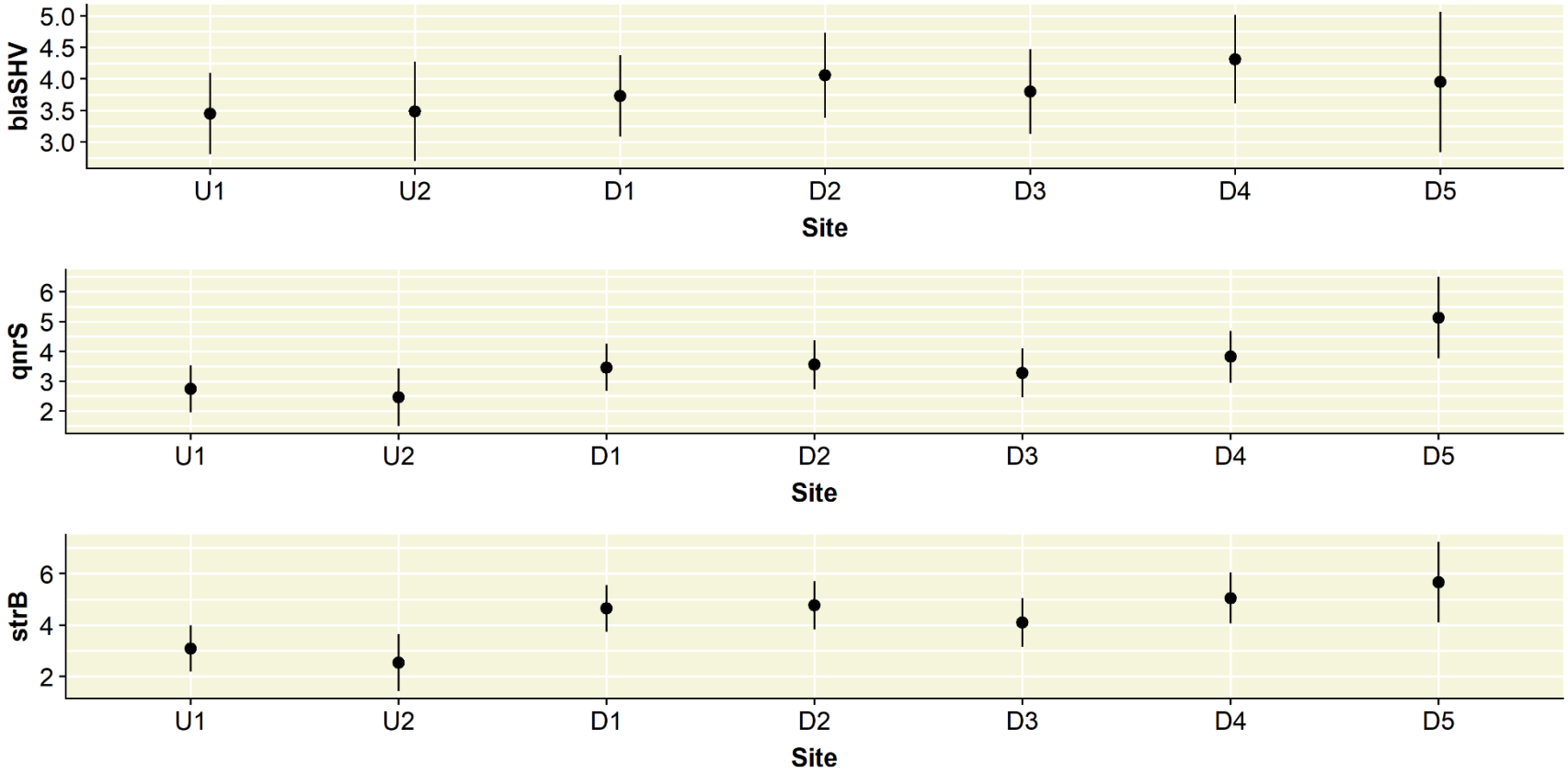


Figure 16. Estimated marginal means and (and standard errors bars) for gene abundance from the linear mixed models for several genes.



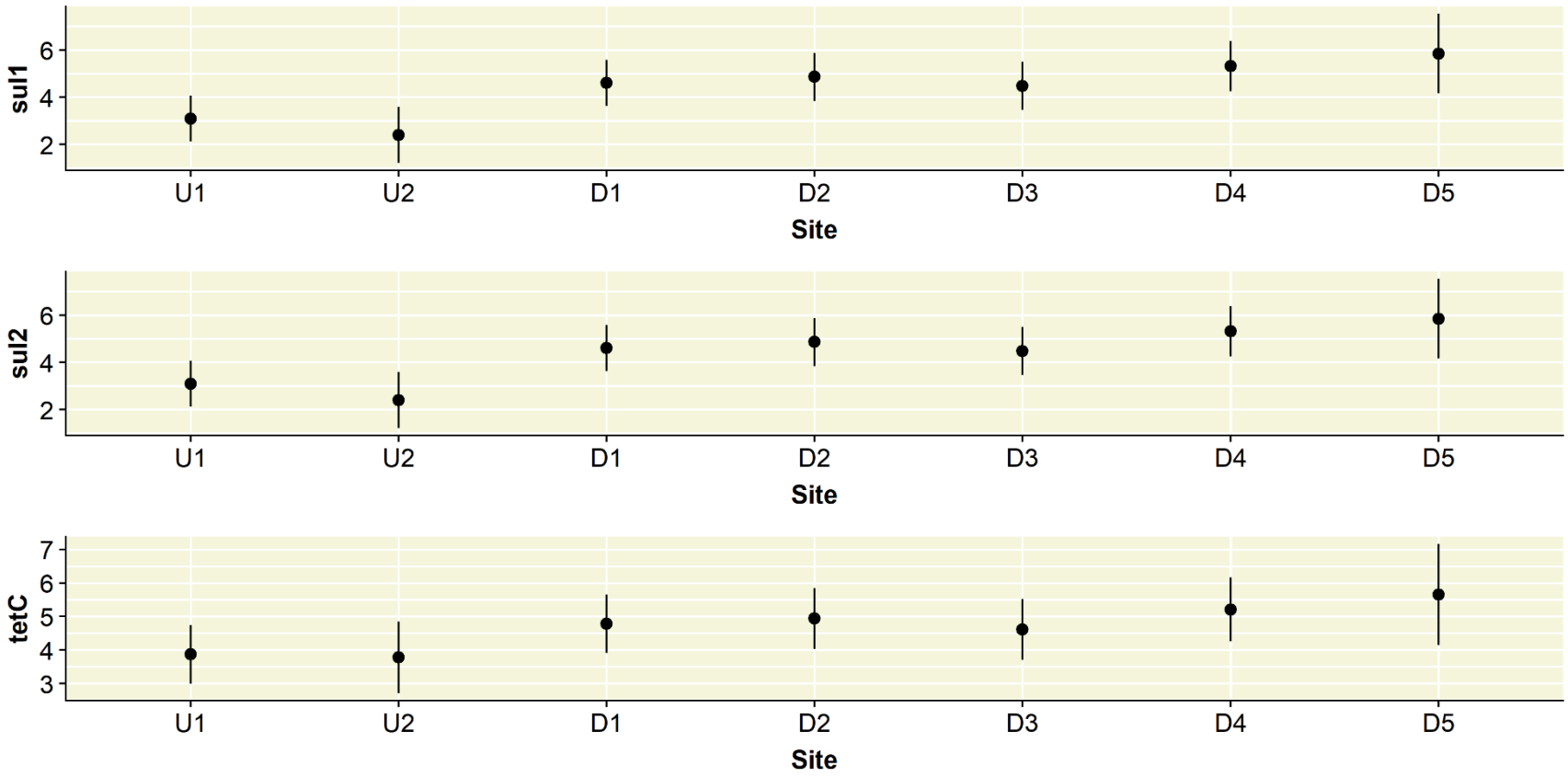

sul1
6
4
2
U1
U2
D1
D2
D3
D4
D5
Site
sul2
6
4
2
U1
U2
D1
D2
D3
D4
D5
Site
tetC
7
6
5
4
3
U1
U2
D1
D2
D3
D4
D5
Site

Figure 17. Antibiotic resistance gene (ARG) abundance for the different bird species across all time points (gene copies per gram feces).

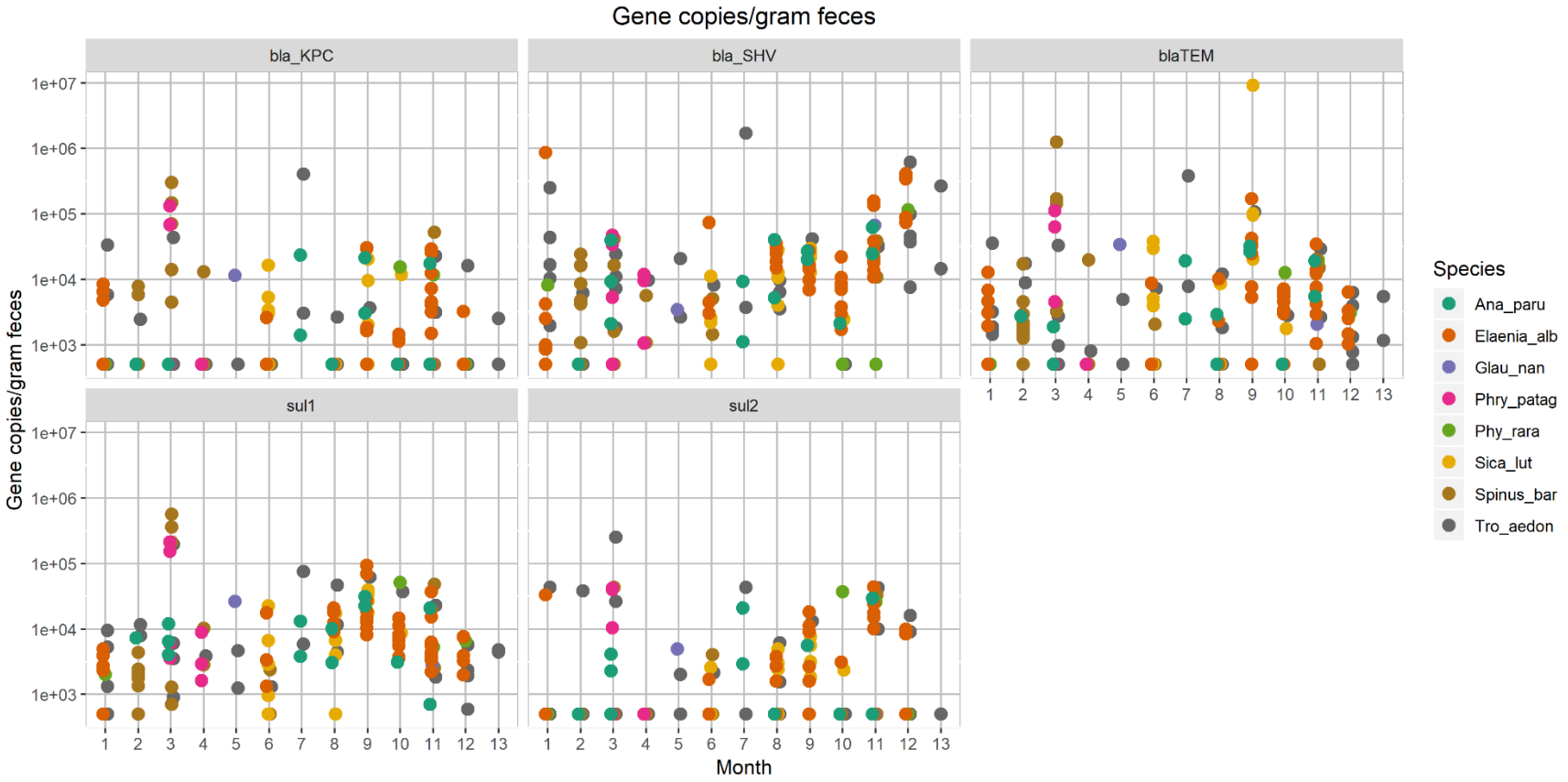


Figure 18. Antibiotic resistance gene (ARG) abundance for the different bird species across all time points (gene copies per copy of 16S rRNA).

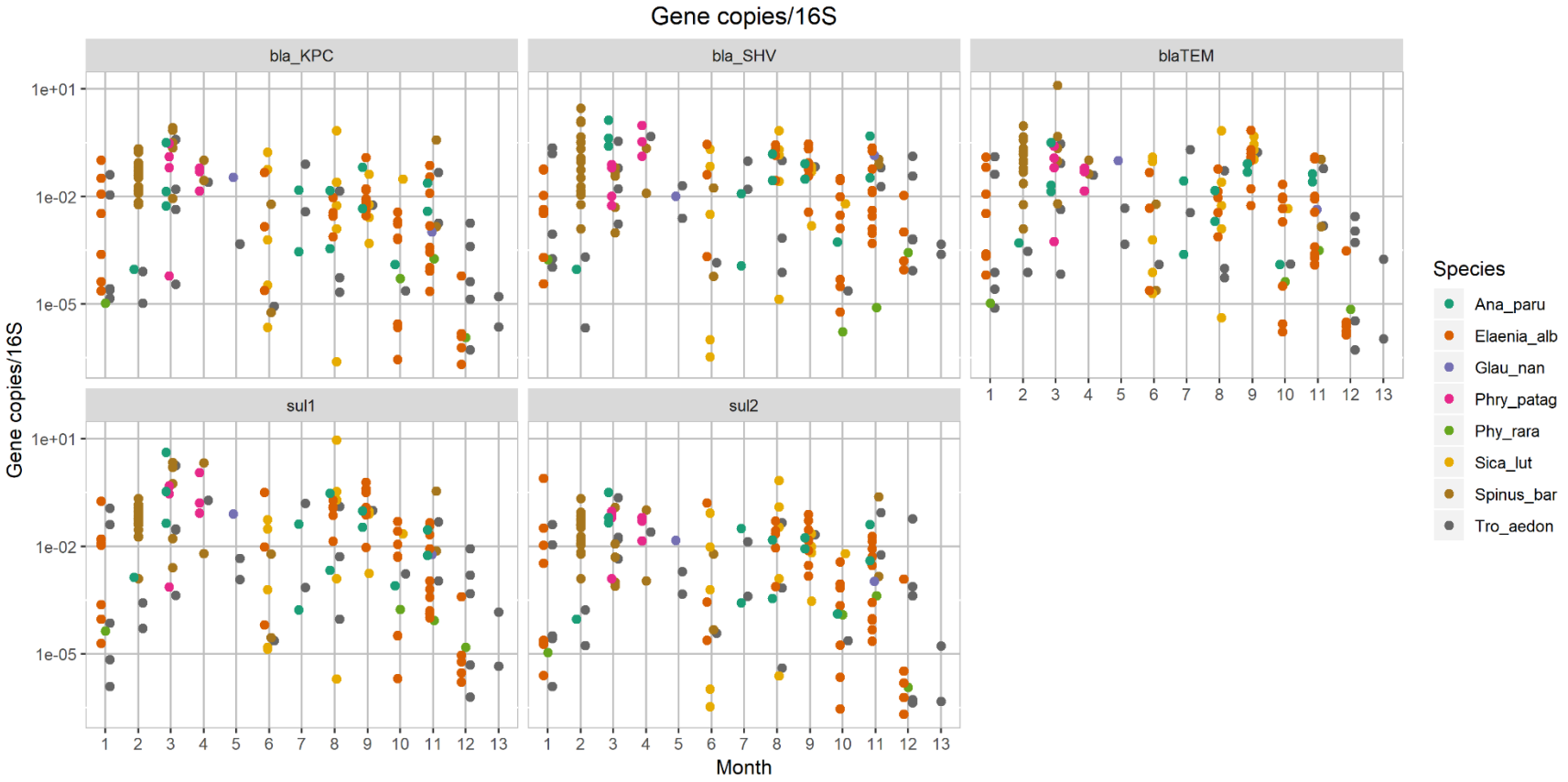


Figure 19. Variable plot from the Principal Component Analysis (PCA) for the bird samples.

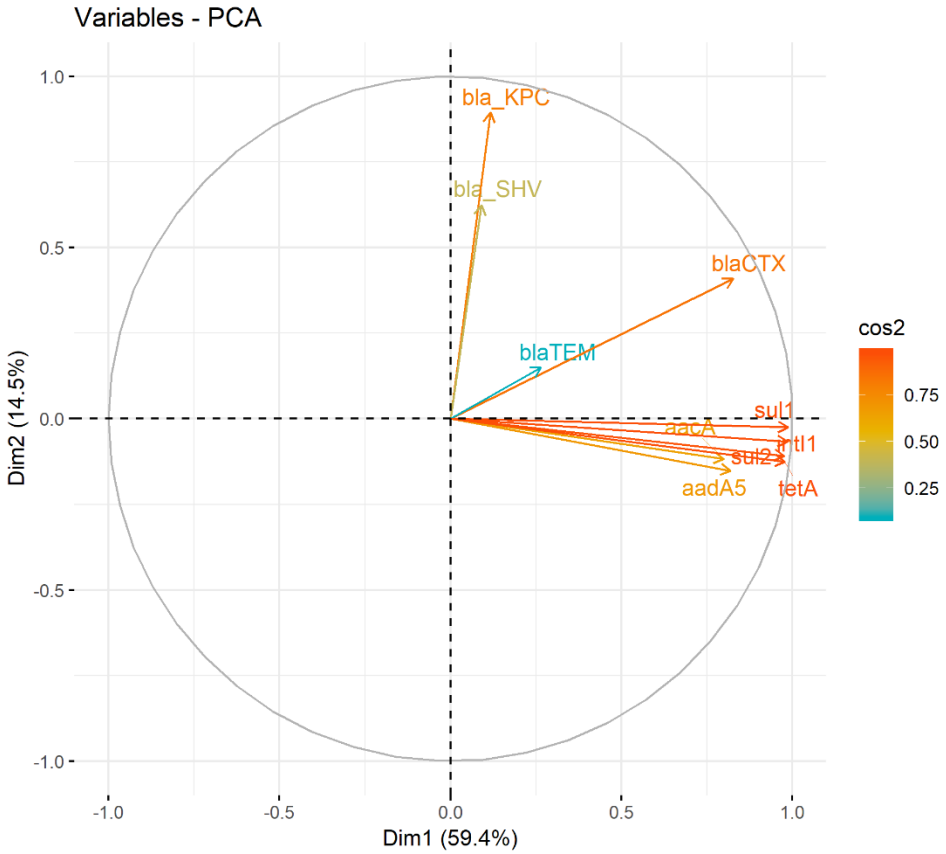


Figure 20. Linear discriminant analysis plot results (absolute scale). M: Migratory birds; R: Resident birds; W: Water.

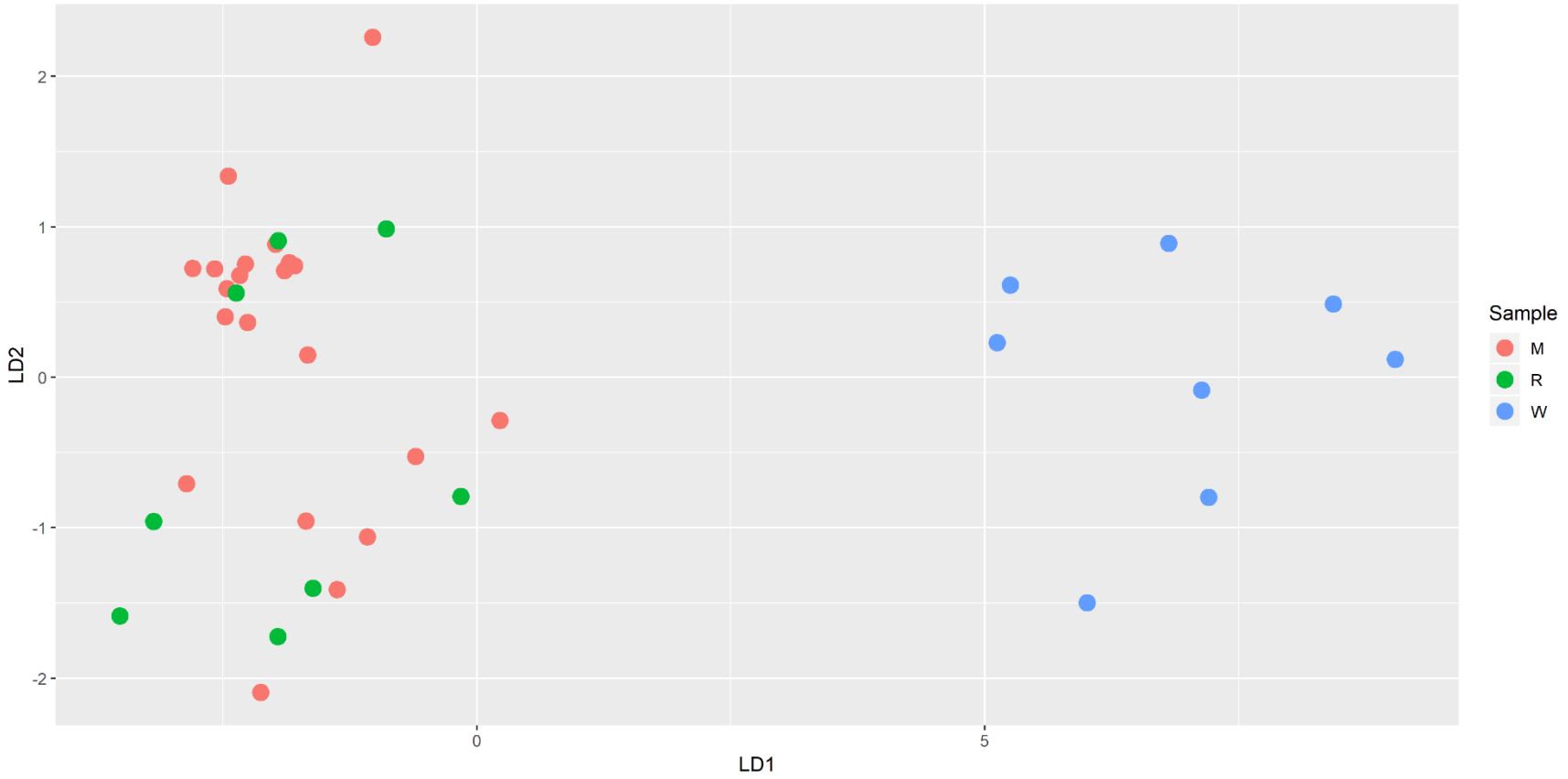
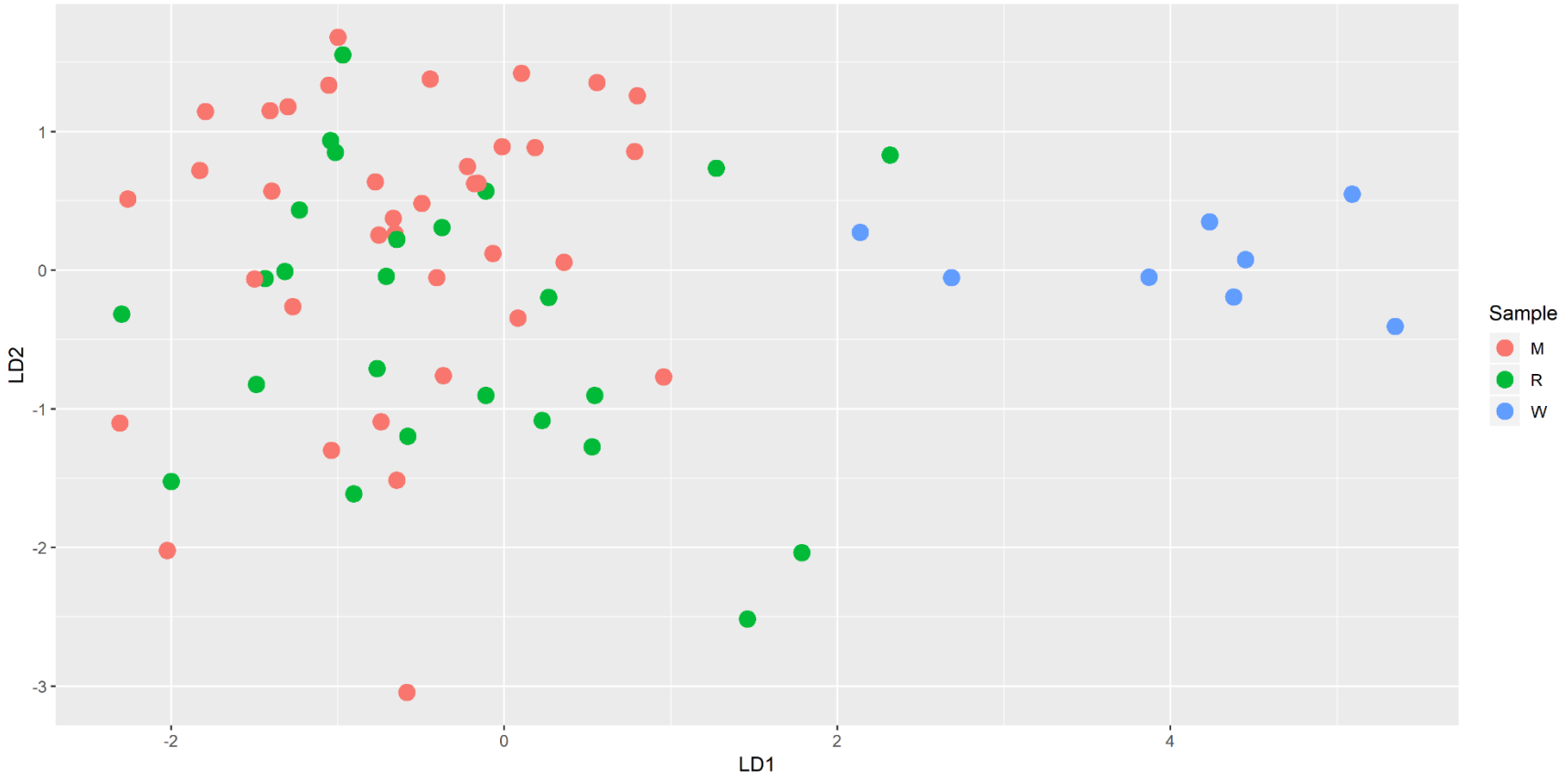


Figure 21. Linear discriminant analysis plot results (relative scale). M: Migratory birds; R: Resident birds; W: Water.



Chapter 6. General Summary and Conclusions

Goal and Justification

A major gap in our understanding of antimicrobial resistance (AMR) is the link between antibiotic resistance found in the natural environment and human and animal health outcomes. To be able to fill in this gap it is crucial to evaluate the spread of resistance through the environment from different sources. The discipline of Epidemiology can provide the methods and tools needed to advance in the field of environmental AMR. This work was therefore carried out with the goal of improving study designs for measuring environmental AMR and to improve our ability to attribute environmental findings to specific point sources.

Summary

First, two systematic literature reviews were conducted to assess the role of point sources on the dissemination of antibiotic resistant bacteria (ARB) (**Chapter 2**) and antibiotic resistance genes (ARG) (**Chapter 3**). Even though the outcomes were divided into ARB and ARG, the conclusions from both reviews highlighted the lack of quantitative causal research on the association of point sources and increases in ARB and ARG in the environment, and the need for improved study design, control of biases, and analytical tools to provide effect measure estimates. Thus, recommendations for environmental AMR studies drawn from the two systematic reviews included: a clear definition for the research question, longitudinal study designs with adequate comparison groups, transparent sampling strategies and field protocols, appropriate spatial scales and data analysis plan for specific research questions, a combination of molecular tools (culture-

independent) and culture-dependent methods, data analysis and data reporting including information about potential confounders and use of analytical methods to control for bias when needed (e.g. regression analysis, spatial analysis) as well as reporting effect measures and measures of variability to quantify the effect of a source on levels of ARB and ARG in the environment.

The conclusions and recommendations from the systematic literature reviews were used to inform the other two chapters (**Chapters 4 and 5**). In an attempt to improve the gaps highlighted by the systematic reviews, two longitudinal studies assessing the role of point sources on environmental levels of ARG were conducted in a watershed system in Chile, South America. This system was chosen because of previous preliminary work in the area assessing other point sources, and because of the critical watershed value (water is used for irrigation, as drinking source, for animal farming, and the watershed hosts important wildlife conservation areas). Two types of point sources were evaluated (three wastewater treatment plants (WWTPs) and five freshwater trout farms), all within the Valdivian watershed (Región de los Rios, Chile). One further outstanding observation from the systematic reviews that was encountered when designing the studies in Chile was the lack of harmonization across methods (field, laboratory and data analysis methods) for environmental AMR studies. Thus, the two studies in Chile were designed and implemented based on the best information and resources available.

The main features of these studies were: their longitudinal nature (sampling the same sites over time), the tight spatial scale to decrease the influence of confounders, quantification of ARG outcomes using a microfluidic-qPCR platform, and use of statistical analysis to assess the influence of the trout farms and WWTPs on ARG levels

in the environment. The microfluidic qPCR approach has only been used in a handful of studies, and only in a few of them to specifically quantify ARG. Despite challenges such as high number of non-detects and data analysis complexity, this approach is very useful for environmental AMR studies as it can simultaneously quantify a large number of ARG with small amount of environmental DNA, which was crucial in this work, especially for bird samples. Other important features were the use of inexpensive equipment for the field work, and a tight collaboration with industry, Academia, and the Ministry of Health. Through the collaboration with Industry it was possible to obtain antibiotic use data from the trout farms which indicated that florfenicol and oxytetracycline were used at the five farms although at different rates between the farms.

Outcome results for both trout farms and WWTPs showed a statistically significant increase of ARG at downstream sites compared to upstream sites. In the case of the trout farms, there was an increase of abundance for *qacG*, *strB*, *sul1*, and several *tet* genes (*tetA*, *tetB*, *tetC*, *tetL*, *tetM*, *tetQ*, *tetS*, *tetW*, *tetX*), and at the WWTPs there was a statistically significant increase downstream of *strB*, *sul1*, and *sul2* abundance. These results indicated that these sources were contributing to releases of ARG into the surrounding environment. However, further downstream dissemination and the biological significance of this increase were not clear. An additional finding for the trout farms was the statistically significant higher abundance of genes at the retention ponds. These findings were consistent with other systems that had found higher ARG abundance downstream from point sources as well as high ARG abundance in cattle and swine lagoons (the equivalent to the trout farm retention ponds), although the gene profile and the quantities varied across the studies. Given the lack of harmonization of environmental

AMR studies as mentioned earlier combined with other differences (geography, types of systems, laboratory methods) it was not entirely possible to compare the findings from the system in Chile with many other published data.

Wild birds were evaluated for their potential to disseminate ARG from one of the WWTPs of the study. They were trapped and sampled (and released again after being banded) for 13 consecutive months from a perimeter around the WWTP. Both migratory and non-migratory species were trapped and out of the 160 birds sampled, only four were captured more than once. The most abundant genes found across all months and across all bird species were: bla_{KPC}, bla_{SHV}, bla_{TEM}, *sul1,* and *sul2.* Migratory birds had a statistically significantly higher abundance for beta-lactamase genes compared to non-migratory species, and when compared to water samples collected from within the WWTP (aeration ponds), there was a clear discrimination between water and bird samples and a small discrimination between migratory and non-migratory bird samples. Even though wild birds are recognized as playing a part in the dissemination of AMR globally due to their feeding behaviors and long distance migrations, it remains a challenge to conduct epidemiologically sound wildlife studies with the goal of attributing ARG abundance to specific sources, mostly due to control group limitations and difficulties in assessing exposures. This topic is worth exploring further, but it should combine tracking technologies to understand bird exposures to different sources, and larger data collections that include birds exposed and not exposed to the source of interest. In addition, further studies should also explore potential health outcomes of AMR for wild birds.

Contribution

Outputs from this dissertation will add to the larger body of knowledge of environmental AMR, especially as examples of what to consider when designing and implementing field studies, and different ways to analyze and report the data. For the specific system in Chile, results from this work will first be communicated to the stakeholders involved in the studies (trout farm managers, wastewater treatment plant managers, Health Department, and academic collaborators). These findings are not likely to have any management repercussions for either the trout farms or the WWTPs. However, further waste management strategies should be considered and evaluated with the goal of reducing the release of ARG into the environment. Also, these results may motivate both education and awareness about watershed sustainability, as well as future research studies in the same watershed to continue the examination of all other point sources on environmental levels of AMR.

Limitations

Despite the contributions of this dissertation, there are also limitations. Systematic reviews, despite being at the top of evidence-based approaches, their main weakness is that of becoming outdated, especially in the case of environmental AMR which is being widely researched nowadays. In the case of the field studies, they lacked power (there were only five farms in one study and three WWTPs in the other, and only a few upstream and downstream sites), there were only four time points, and physical-chemical parameters were not measured and thus not taken into account in the statistical models. There was also lack of a control site for the wild bird study, which could have been potentially helpful in assessing the WWTP as a source of exposure. The

recommendations outlined by the systematic reviews are ideal, but after trying to apply those recommendations through the field studies in Chile, it is even more evident that conducting environmental AMR studies is very challenging.

Future Directions

Relevant methodological advances and study design recommendations from this work are applicable to assess the association between point sources and environmental AMR in any other system. However, a step forward for this field would be to standardize methods as much as possible to be able to compare findings across studies. For the watershed in Chile in particular, a larger scale watershed study could be conducted to evaluate the role of other point sources and assess the relative contribution of each source on the environmental levels of AMR by incorporating spatial modelling and landscape ecological methods. That could be coupled with ecological assessments to evaluate the watershed health over time, and ultimately establish a long-term watershed monitoring system.

More broadly, an area that is in need of more research in this field is an understanding of the different pathways of environmental AMR exposure to humans and animals. Once those routes are well quantified, health consequences for humans and animals can be assessed. Whatever path this field takes, what is imperative in order to make progress is the creation of multidisciplinary teams that include engineers, chemists, public health experts, veterinarians, epidemiologists, microbiologists, ecologists, among others, so that the problem of AMR can be tackled holistically.

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APPENDIX A.

Table 15. Findings for the studies included in this systematic review (ARB) that assessed human waste (WWTP, industrial, urban areas) as a point source, organized by risk of bias (from low to high) (n=35). WWTP: Wastewater treatment plant.

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
Akiyama and Savin (2010)	Proportion of antibiotic resistant *E. coli* isolated from river surface water and from sediment 20 m upstream (M1) compared to 640 m downstream (M2), and 2000 m downstream (M3) of a WWTP discharge point over a 2-year sampling campaign	Low (Small spatial scale)	Disk diffusion (CLSI) / 3 antibiotics (1st year of sampling), 6 antibiotics (2nd year)	In surface water, significantly higher proportion of resistant *E. coli* downstream (M2, M3) compared to M1 during the 1st year of sampling. In the 2nd year, proportion at M2 significantly higher than at M1 only 1/3 of sampling times, and M3 not significantly different than M1. In sediment, significantly higher proportion at M2 compared to M1 for ampicillin and trimethoprim, but higher at M1 compared to M2 for tetracycline; for sulfamethoxazole there was no difference. Statistical inference conducted using split-plot ANOVA ($p<0.05$). Effect esimates were not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
Akiyama and Savin (2010)	Proportion of antibiotic resistant total coliforms isolated from river surface water and sediment 20 m upstream (M1) compared to 640 m downstream (M2), and 2000 m downstream (M3) of a WWTP discharge point over a 2-year sampling campaign	Low (Small spatial scale)	Disk diffusion (CLSI) / 3 antibiotics (1st year of sampling), 6 antibiotics (2nd year)	In surface water, no significant difference between upstream and downstream. In sediment, higher proportion at M2 compared to M1 for sulfamethoxazole and trimethoprim-resistant coliforms, but no significant difference for ampicillin and tetracycline. Statistical inference conducted using split-plot ANOVA ($p<0.05$). Effect esimates were not provided
Leclercq et al. (2013)	Proportion of antibiotic resistant *Enterococci* isolated from river surface water upstream from a WWTP (site 6) compared to a river site immediately downstream from the WWTP (site 5)	Low (Small spatial scale)	Disk diffusion (CA-SFM) / 12 antibiotics	Overall, higher proportion of resistant bacteria downstream compared to upstream. Effect estimates and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
Li et al. (2010)	Prevalence of resistant heterotrophic bacteria isolated from river surface water 5 km upstream from a WWTP treating water from an oxytetracycline production plant compared to a site 20 km downstream from the WWTP	Low (Authors acknowledge the WWTP is the only source in the spatial scale they compared)	Disk diffusion (CLSI) / 10 antibiotics	Significantly higher prevalence at downstream site compared to upstream site (Mann Whitney U test, $p<0.01$). Effect estimates were not provided
Marti et al. (2014)	Proportion of resistant *Pseudomonas spp.*, *Cytophage-Flavobacterium spp.*, and coliforms isolated from river sediment and biofilm collected over 2 campaigns (June and September) 100 m upstream from a WWTP compared to a site 100 m downstream from the WWTP	Low (Small spatial scale)	Disk diffusion (CLSI) / Ciprofloxacin	For all types of bacteria types in sediment samples: higher proportion downstream in June and higher upstream in September. Same trend in biofilm samples, except for *Pseudomonas spp.* isolates (higher proportion upstream both time periods). Effect estimates and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
Osińska et al. (2016)	Prevalence of fluoroquinolone-resistant bacteria (FQRB) isolated from river surface water 600 m upstream from a WWTP compared to resistant isolates at 600 m downstream from the WWTP	Low (Small spatial scale)	Disk diffusion (EUCAST) / 9 antibiotics	Higher prevalence downstream compared to upstream (40% vs 32%). Effect estimates and accompanying statistical inference were not provided
Schreiber and Kistemann (2013)	Proportion of resistant *Rhodospirillaceae* isolated from surface water 80 m upstream compared to 80 m downstream from a WWTP	Low (Small spatial scale)	Disk diffusion (DIN 58940-3) / 9 antibiotics	Resistance in *Rhodospirillaceae* did not appear to be associated to wastewater discharge. There was no statistical correlation between resistance levels at the sampling sites and influence of wastewater (Cramer-V correlation). Effect estimates were not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
Sidrach-Cardona et al. (2014)	Prevalence of resistant *E. coli* isolated from river surface water and from sediment 50 m upstream from a WWTP (site 3), a site immediately downstream from the WWTP (site 4), a site about 50 m downstream (site 5), and a site 500 m downstream from the WWTP (site 6)	Low (Unlike introductio n of any type of bias)	Disk diffusion (NCCLS) / 7 antibiotics	For most antibiotics, prevalence of resistant *E. coli* was higher at downstream sites compared to upstream sites in both sediment and water samples for azithromycin, doxycycline, streptomycin, and tetracycline. Prevalence was higher upstream compared to downstream for ampicillin in both sediment and water samples. For penicillin and erythromycin, there was a 100% prevalence at all sites in both types of samples. Effect estimates and accompanying statistical inference were not provided
Sidrach-Cardona et al. (2014)	Prevalence of resistant *E. coli* isolated from river surface water and from sediment 50 m upstream from an Antibiotic Production Plant (APP) and at a site 100 m downstream from the APP	Low (Unlike introductio nof any type of bias)	Disk diffusion (NCCLS) / 7 antibiotics	Prevalence of resistant *E. coli* in water samples was either higher downstream, or the same for both sites except for doxycycline, tetracycline, and streptomycin, where it was higher upstream. In sediment samples, prevalence tended to be either higher or the same upstream and downstream, except for

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
				doxycycline. Effect estimates and accompanying statistical inference were not provided
Alharbi (2012)	Concentration of *MRSA* isolated from soil compared at different distances (10 m, 500 m and 2000 m) from hospital facilities in both Saudi Arabia and the UK	Unclear (Not enough information provided about sample collection and potential influence of other sources)	Disk diffusion / (Not reported) / Methicilin	Qualitative analysis did not suggest a relationship between distance to the hospital facilities and concentration of *MRSA* in soil samples at any of the sites. Effect estimates and accompanying statistical inference were not provided
Amador et al. (2015)	Proportion of resistant *Enterobacteriaceae* bacteria isolated from river water collected 500 m downstream (DRW) compared to 500 m upstream (URW) of WWTP discharge	Unclear (Lack of information regarding other potential sources)	Disk diffusion (CLSI) / 13 antiobiotics	Evidence is conflicting for an impact of the WWTP on the proportion of resistant *Enterobacteriaceae* in river water based on qualitative comparison. Effect estimates and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
Czekalski et al. (2012)	Proportion of several types of resistant bacteria isolated from lake sediment and lake surface water immediately adjacent to STEP (WWTP outfall site) compared to measurements at DP (3.2 km away from STEP)	Unclear (Insufficient information about possible confounding due to lake depth)	Disk diffusion (DIN, EUCAST) / Three combinations of antibiotics at inhibitory concentrations: (a) sulfamethoxazole, trimethoprim and streptomycin, (b) norfloxacin and ceftazidime, (c) clarithromycin and tetracycline	In surface water, the proportion resistant for all bacteria type was low (<1%) or absent at both lake sites. In sediment, the proportion resistant was low (< 1%) or absent at both lake sites except for antibiotic combination a), but the evidence of any effect was inconclusive. Effect estimates and accompanying statistical inference were not provided
Fincher et al. (2009)	Prevalence of resistant *E. coli* O157:H7 isolated from river surface water compared between the most upstream site (site 1), a site just upstream from a city (site 2), and downstream from the city (site 3)	Unclear (Not enough information provided about the potential impact from farms	Disk diffusion (Manufacturer) / 8 antibiotics	There was little support for an effect of the city on the prevalence of resistant *E. coli* 0157:H7 in water samples. At site 3 there were 2/21 (9.5%) isolates that showed resistance to 3 antibiotics at the same time compared to 0 at site 2 and 1/21 (4.8%) at site 1. For isolates resistant

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
		near the city)		to 2 antibiotics, site 3 had 9.5% compared to 0 at the upstream sites. For isolates resistant to just 1 antibiotic, there was a 9.5% prevalence at site 2 compared to 0 at sites 1 and 3. Effect estimates and accompanying statistical inference were not provided
Fuentefria et al. (2008)	Proportion of resistant *Pseudomonas aeruginosa* isolated from surface water compared between point B (upstream from a hospital) and point C (downstream from the hospital effluent). No distance between these sites was reported	Unclear (No information provided about distance between sampling locations)	Disk diffusion / (CLSI) 11 antibiotics	Overall, proportion of resistant *Pseudomonas aeruginosa* was higher downstream compared to upstream (55.6% vs 22.7%). Effect estimates and accompanying statistical inference were not provided
Fuentefria et al. (2011)	Proportion of resistant *Pseudomonas aeruginosa* isolated from surface water compared between a site 800 m upstream (W1) and 600 m downstream	Unclear (Not enough information provided about other	Disk diffusion (CLSI) / 11 antibiotics	Higher proportion of resistant *Pseudomonas aeruginosa* upstream compared to downstream for imipenem (20.8% vs 5.5%), but higher downstream compared to upstream for meropenem (22.2% vs

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
	(W2) from the discharge of HSVP hospital	potential sources)		0%). Not detected for the other antibiotics. Effect estimates and accompanying statistical inference were not provided
Fuentefria et al. (2011)	Proportion of resistant *Pseudomonas aeruginosa* isolated from surface water compared between a site 100 m upstream (W3) and 100 m downstream (W4) from HDP hospital	Unclear (Not enough information provided about other potential sources)	Disk diffusion (CLSI) / 11 antibiotics	Higher prevalence upstream compared to downstream for both imipenem (97.7% vs 96.7%) and meropenem (2.3% vs 0%). Not detected for the other antibiotics. Effect estimates and accompanying statistical inference were not provided
Gallert et al. (2005)	Proportion of resistant *Enterococci*, fecal coliforms, and *Pseudomonads* isolated from groundwater downstream from a leaky sewer at different distances from the source (1 m, 1.8 m, 2 m, 15 m)	Unclear (Not enough information provided about other potential sources)	Disk diffusion (Guidelines not reported) / 14 antibiotics	No evidence for an effect of the leaky sewer on the proportion of any of the types of resistant bacteria by distance. Effect estimates and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
	and at 410 m away (reference site)			
Harnisz (2013)	Prevalence of resistant heterotrophic bacteria isolated from surface water 600 m upstream from a WWTP compared to a site 600 m downstream from the WWTP	Unclear (Not enough information provided about other potential sources)	Disk diffusion (CLSI) / 6 antibiotics	All resistant isolates showed higher prevalence downstream compared to upstream except for CEF^R (28% downstream vs 47% upstream). ENR^R and DOX^R were barely detected. Effect estimates and accompanying statistical inference were not provided
Koczura et al. (2012)	Proportion of resistant *E. coli* isolated from river surface water upstream from a WWTP compared to a downstream site from the WWTP (no specific distance reported)	Unclear (Not enough information provided about the spatial scale of the study)	Disk diffusion (CLSI) / 27 antibiotics	For most resistant *E. coli* isolates there was a higher proportion downstream compared to upstream except for isolates resistant to amikacin and streptomycin that had a higher prevalence upstream compared to downstream. For some antibiotics there was a statistically significant relationship (Pearson Chi-square test, $p<0.05$), but effect estimates were not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
Li et al. (2009)	Prevalence of resistant heterotrophic bacteria isolated from river surface water at a point downstream 30 km from a WWTP treating water from a Penicilin G production plant, and an upstream point 5 km from the WWTP	Unclear (Large spatial scale but authors acknowledge being not aware of any other point sources in that area)	Disk diffusion (CLSI) / 18 antibiotics	Prevalence downstream higher than upstream for all antibiotics except for ciprofloxacin and levofloxacin. Effect estimates and accompanying statistical inference were not provided
Li et al. (2011)	Proportion of resistant isolates from different bacteria phyla from river surface water 5 km upstream compared to a site 30 km downstream from a WWTP treating Penicilin G wastewater	Unclear (Not enough information provided about potential effect of other sources)	Disk diffusion (Guidelines not reported) / Ampicillin	Higher proportion found downstream compared to upstream (about 65% of isolates resistant to ampicillin vs <10% upstream). Effect estimates and accompanying statistical inference was not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
Li et al. (2011)	Proportion of resistant isolates from different bacteria phyla at an upstream site (5 km from a WWTP that receives oxytetracycline production wastewater) compared to a downstream site (20 km from the WWTP)	Unclear (Not enough information provided about potential effect of other sources)	Disk diffusion (Guidelines not reported) / Oxytetracycline	Higher proportion found downstream compared to upstream (about 55% of isolates resistant to oxytetracycline vs <5% upstream). Effect estimates and accompanying statistical inference was not provided
Oberle et al. (2012)	Proportion of resistant *E. coli* isolated from river surface water at a site upstream from a WWTP compared to a river site immediately downstream from the WWTP discharge point	Unclear (Not enough information provided about potential effect of agricultural sources and untreated waste in the area)	Disk diffusion (French National Guidelines) / 11 antibiotics	Higher proportion of resistant *E. coli* downstream compared to upstream for isolates that were resistant to at least 1 antibiotic (26% vs 22.2%), and for multidrug resistance (14% vs 11.1%). Effect estimates and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
Reinthaler et al. (2003)	Proportion of resistant *E. coli* isolated from river surface water at a site 100 m upstream from a WWTP (A) compared to a site 100 m downstream from WWTP (A)	Unclear (No information provided about locations and potential influence from other sources)	Disk diffusion (CLSI) / 24 antibiotics	Proportion of resistant *E. coli* higher upstream compared to downstream for the majority of antibiotics except for tetracycline and trimethroprime /sulfamethoxazole, where the proportion was higher downstream compared to upstream. Effect estimates and accompanying statistical inference were not provided
Reinthaler et al. (2003)	Proportion of resistant *E. coli* isolated from river surface water at a site 100 m upstream from a WWTP (B) compared to a site 100 m downstream from WWTP (B)	Unclear (No information provided about locations and potential influence from other sources)	Disk diffusion (CLSI) / 24 antibiotics	Proportion of resistant *E. coli* higher downstream compared to upstream for the majority of antibiotics except for piperacillin, nalidixic acid, tetracycline, and nitrofurantoin, where proportion was higher upstream compared to downstream. Effect estimates and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
Reinthaler et al. (2003)	Proportion of resistant *E. coli* isolated from river surface water at a site 100 m upstream from a WWTP (C) compared to a site 100 m downstream from WWTP (C)	Unclear (No information provided about locations and potential influence from other sources)	Disk diffusion (CLSI) / 24 antibiotics	No clear evidence for an impact of WWTP in river samples, being the proportion of resistant *E. coli* similar upstream and downstream. Effect estimates and accompanying statistical inference were not provided
Rees et al. (2015)	Proportion of resistant *E. coli* isolated from shellfish at different sampling points as the continuous exposure defined as standardized seaway distance to the nearest human point source	Unclear (Not enough information about the other sources in the area)	Microdilution (CLSI) / 14 antibiotics	In general, the mixed-model found that ARB positive isolates had a lower proportion of agricultural land adjacent compared to non-ARB isolates. Overall, resistant isolates were significantly closer to human point sources. Mean seaway distance to nearest human source in ARB isolates (and standard deviation) was 1247± 802.0 (n=6), and the mean seaway distance in non-resistant isolates was: 4286.6± 3302.1 (n=16). The model accounted for the proportion of surrounding land in agricultural use and for correlation due to sampling, site, month, and

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
				shellfish species. Effect estimates were not provided
Sadowy and Luczkiewicz (2014)	Prevalence of resistant *Enterococcus faecalis* isolated from surface water from a site near the marine outfall of a WWTP compared to a river site (farther from the WWTP)	Unclear (Not enough information about other potential sources)	Disk diffusion (CLSI) / 11 antibiotics	Prevalence of resistant *E. faecalis* higher at the marine outfall site compared to the river site, except for quinupristin-dalfopristin (prevalence at the river site higher). Effect estimates and accompanying statistical inference were not provided
Sadowy and Luczkiewicz (2014)	Prevalence of resistant *Enterococcus faecium* isolated from surface water from a site near the marine outfall of a WWTP compared to a river site (farther from WWTP)	Unclear (Not enough information about other potential sources)	Disk diffusion (CLSI) / 11 antibiotics	There was little support for an effect of the WWTP on the prevalence of resistant *E. faecium.* There were no resistant isolates for most of the antibiotics tested at both sampling sites. Only ciprofloxacin (not detected at the marine outfall and 40% prevalence at the river site), and erythromycin (100% marine site vs 20% river site) were detected. Effect estimates and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
Topić Popović et al. (2015)	Proportion of resistant heterotrophic bacteria isolated from river surface water compared between a site downstream (site 9) from a WWTP and a reference site upstream from the WWTP (site 1)	Unclear (Not enough information provided about sampling design and influence of confounders)	Disk diffusion (Manufacturer's guidelines) / Oxytetracycline	There was no resistant isolates in site 1 (reference) and 100% resistant isolates in site 9 (downstream). Effect estimates and accompanying statistical inference were not provided
West et al. (2011)	Prevalence of resistant fecal coliforms isolated from surface water upstream and downstream from a WWTP (1-1.5 km from the WWTP in each direction)	Unclear (Information provided about presence of other sources but unknown impact at the spatial	Disk diffusion (Study defined) / 5 antibiotics	For ampicillin resistance, prevalence of resistant coliforms (%) was higher downstream compared to upstream. For MDR, prevalence (%) was lower downstream compared to upstream. However, neither of these differences were statistically significant (Fisher's exact test; α=0.05). Effect estimates were not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
		scale of the study)		
Xu et al. (2012)	Proportion of resistant coliform bacteria isolated from river surface water upstream and downstream from a WWTP (no distance reported)	Unclear (Not enough information provided about spatial scale)	Microbroth dilution (CLSI) / 7 antibiotics	Higher proportion downstream compared to upstream for all antibiotics tested, except for ciprofloxacin. Effect estimates and accompanying statistical inference were not provided
Zhang et al. (2015)	Proportion of resistant fecal coliforms isolated from river surface water from a downstream site (site E) compared to an upstream site (site D), 200 m upstream from a WWTP	Unclear (Site D likely heavily influenced by other urban outfalls of sewage and storm water, but not enough information provided)	Plating method (CLSI) / Tetracycline	Qualitatively, proportion of tet-resistant coliforms lower downstream compared to upstream. Effect estimates and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
Zhang et al. (2015)	Among phenotypically-tetracycline-resistant fecal coliforms isolated from river water, proportion of isolates with selected resistance genes from a site downstream (E) of a WWTP compared to proportion at site 200 m upstream (site D)	Unclear (Site D likely heavily influenced by other urban outfalls of sewage and storm water, but not enough information provided)	*tetA* *tetB* *tetC* *tetG* *tetK* *tetM* *tetO* *tetQ* *tetX*	Qualitatively, evidence did not support an effect of the WWTP on the proportion of tetracycline-resistant fecal coliforms carrying specific resistance genes downstream. No apparent difference in proportion of isolates carrying *tetA, tetC, tetQ, tetX* between downstream and upstream sites. Proportion carrying *tetB, tetK* lower downstream. Results for *tetG, tetM, tetO* not reported. Effect estimates and accompanying statistical inference were not provided
Abia et al. (2015)	Prevalence of resistant *E. coli* isolated from river surface water compared across several sites: DAS (downstream of Daspoort WWTP), AP1 (downstream of DAS), AP6 (downstream of AP1), A5 (downstream of AP6 - tributary), A4 (downstream of A5), A3 (downstream of A4), Rooiwal WWTP	High (Large spatial scale with several WWTP and other sources present; no control for confounding)	Disk diffusion (CLSI) / Chloramphenicol	No clear trend found. Effect estimates and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
	(downstream of AP1), AP2 (downstream of Rooiwal WWTP), AP7 (downstream of AP2 and upstream of Temba WWTP), Babelegi WWTP, AP8 (downstream of Babelegi) and AP9 (downstream of AP8)			
Blaak et al. (2014)	Prevalence of ESBL-producing *E. coli* isolated from surface water upstream from a WWTP, at the discharge point, and downstream from 4 different WWTP in 4 different regions, each one of the WWTP located 1-2 km from the downstream site	High (Likely influence from other sources)	Disk diffusion (CLSI) / 12 antibiotics	Across the 4 regions, prevalence was highest at the upstream sites compared to the discharge points and to the downstream sites for 5 antibiotics: ceftazidime, ciprofloxacin, tetracycline, streptomycin, and chloramphenicol. Prevalence was highest at the discharge point compared to upstream and downstream sites for nalidixic acid, gentamycin, trimethoprim, and sulfoxizole. Prevalence was highest at the downstream site compared to the discharge point and the upstream site for coamoxiclav, and the same

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
				prevalence (100%) at the downstream and discharge point for ampicillin (vs 98% for the upstream site), and a 100% at all sites for cefotaxime. Across the 4 regions, the prevalence of MDR among ESBL-producing isolates was: 63% at the WWTP discharge point; 62% upstream; and 41% at the downstream. Effect estimates and accompanying statistical inference were not provided
Goni-Urriza et al. (2000)	Proportion of resistant *Enterobacteriacea* isolated from river surface water compared between a sewage dump (Kp 0), a site upstream (Azazuri, at 0.5 km), and 5 sites downstream at 10, 16, and 30 km distance	High (Likely influence from other sources)	Disk diffusion (French National guidelines) / 22 antibiotics	Higher proportion downstream compared to upstream. Greatest increase with beta-lactams (0% upstream to 20.5% downstream) and tetracycline (12.5% to 24.3%). Effect estimates and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
Goni-Urriza et al. (2000)	Proportion of resistant *Aeromonas spp.* isolated from river surface water compared between a sewage dump (Kp 0), a site upstream (Azazuri, at 0.5 km), and 5 sites downstream. at 10, 16, and 30 km distance	High (Likely influence from other sources)	Disk diffusion (French National guidelines) / 22 antibiotics	Higher prevalence downstream compared to upstream. Tetracyclines had the highest difference (0% to 27.5%) and co-trimoxizazole (0 to 26.6%). Effect estimates and accompanying statistical inference were not provided
Kotlarska et al. (2015)	Prevalence of resistant *E. coli* isolated from surface water from a WWTP discharge point (marine outflow) of WWTP (A) compared to a river site 2.5 km away	High (River likely impacted by numerous upstream activities)	Microdilution (EUCAST) / 17 antibiotics	For most antibiotics, marine outflow showed higher prevalence of resistant *E. coli* than at the river site, and it was statistically significant for ampicillin, trimethoprim-sulfamethoxazole, ciprofloxacin, and levofloxacine (Fisher exact test, $p<0.05$). Although non-statistically significant, there was a higher prevalence at the river site compared to the marine outflow for gentamicin and cefazolin. Effect estimates were not provided
Laroche et al. (2009)	Proportion of resistant *E. coli* isolated from river surface water upstream (KP202) compared to a	High (Several other WWTP	Disk diffusion (French National guidelines) / 16 antibiotics	There was a higher proportion of resistant *E. coli* downstream compared to upstream. For resistance to at least 1 antibiotic, the geometric

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
	site about 30 km downstream (KP 260) from a WWTP pooled across several time points	and other point sources that could influence the results)		mean across all sampling times was 49.6% (31.0-68.7) at KP260 (downstream) and 30.2% (16.0-50.0) at KP202 (upstream). For resistance to more than 1 antibiotic, the geometric mean of all sampling times was 28.1% at KP260 and 21.1% at KP202. Effect estimates and accompanying statistical inference were not provided
Marinescu et al. (2015)	Proportion of resistant Gram negative bacteria (several species but not differentiated in the analysis by sites) isolated from river surface water 1000 m upstream and 200 m downstream from a WWTP	High (Pooling lac+ and lac- isolates seems likely to introduce bias if lac +/- status is associated both with the particular sampling site and	Disk diffusion (CLSI) / 18 antibiotics	Higher proportion in the downstream site compared to the upstream site for all antibiotics, some of them statistically significant by One-way ANOVA ($p<0.001$). There was no detection of resistant bacteria to ticarcylin and aztreonam. No effect estimates provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
		with resistance)		
Marinescu et al. (2015)	Among phenotypically-resistant Gram-negative bacterial isolates (predominantly *Enterobacteriaceae*) from river water, proportion of isolates hosting selected resistance genes from river water sampled at a site 200 m downstream of a WWTP compared to a site 1000 m upstream	High (Pooling lac+ and lac- isolates seems likely to introduce bias if lac +/- status is associated both with the particular sampling site and with resistance)	*bla*^{TEM} *bla*^{SHV} *bla*$^{CTX-M}$ *bla*^{CMY} *bla*^{NDM} *bla*$_{VIM}$ *bla*$_{IMP}$ *dfrA1-aadA1* *intl1* *qnrA* *qnrB* *qnrS* *sul1* *sul2* *tetA* *tetB* *tetC* *tetD* *tetM*	Overall, there was some evidence for an association between WWTP impact and mechanism of resistance for some genes. For beta-lactamase genes, *bla*$_{NDM}$ and *bla*$_{CMY}$ were detected in 100% and 25%, respectively of isolates that exhibited resistance to beta-lactams from the downstream samples compared to 0% isolates upstream, while the remaining *bla*-type genes were not detected in isolates at either site. For non-beta-lactamase genes, *tetD, sul1*, and *qnrB* were detected in 100%, 20%, and 100%, respectively of isolates that exhibited acquired resistance phenotypes from the downstream samples compared to 0% isolates upstream, while the remaining genes were not detected in isolates at either site.

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
				Effect estimates and accompanying statistical inference were not provided for these comparisons
Mondragón et al. (2011)	Proportion of resistant *Enterococcus spp.* isolated from river surface water at a reference site (AT) and at a downstream site (PP) from the point source (treated sewage site, PL)	High (Likely influence of other sources)	Disk diffusion (CLSI) / 5 antibiotics	Higher proportion of resistant *Enterococcus spp.* downstream compared to the reference site for ampicillin, vancomycin, and gentamicin, but higher at the reference site for kanamycin, and 100% resistant for ciprofloxacin at both sites. Effect estimates and accompanying statistical inference were not provided
Oh et al. (2009)	Proportion of resistant oligotrophic and heterotrophic bacteria isolated from river surface water upstream (US1, US2) and downstream (DS1, DS2, DS3) from a WWTP	High (Large spatial scale)	Disk diffusion, 2 different nutrients (R2A and LB), CLSI / Tetracycline, Vancomycin	For tetracycline, there were no detection of resistant isolates. For vancomycin, there was a difference in the proportion depending on the nutrient used. For R2A, the proportion was highest at DS2 and lowest at US1. For LB, the proportion was highest at US1 and lowest at DS2. Effect estimates and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	Detection method / Antibiotics	Relevant findings
Suzuki et al. (2013)	Proportion of resistant *Pseudomonas aeruginosa* isolated from surface water upstream, midstream (near a city), and downstream from the city (no distances specified)	High (Upstream site influenced by other sources)	Microbroth dilution (CLSI) / 10 antibiotics	

Table 16. Findings for the studies included in the systematic review (ARB) that had animal agriculture (both terrestrial and aquaculture) as a point source, presented by risk of bias (from low to high) (n=12).

Citation	Relevant comparison	Overall risk of bias	ARB / Detection method/ Antibiotics	Relevant findings
Harnisz et al. (2015)	Prevalence of resistant heterotrophic bacteria (oxytetracycline OTC^R and doxycycline DOX^R isolates) from surface water upstream (URW) and downstream (DRW), about 200 m distance each from a fish farm effluent	Low (Small spatial scale)	Disk diffusion (CLSI) / 12 antibiotics	For OTC^R, prevalence was higher upstream compared to downstream, except for norfloxacin, enrofloxacin, trimethoprim/sulfamethoxazole, and for tetracycline. For DOX^R, prevalence was higher downstream compared to upstream, except for cefotaxime and for those antibiotics where the prevalence was the same at both sites (amoxicillin+clavulanic acid, and tetracyline). Effect estimates and accompanying statistical inference were not provided
Li et al., (2015b)	Prevalence of resistant *E. coli* isolated from groundwater in wells near-dairy farms (less than 2.4 km) and from wells far from the farms (non-dairy)	Low (Small spatial scale and no other likely sources influencing the comparison in that scale)	Sensititre (CLSI) / Not reported	At the sites near-dairy, from 5 isolates tested, 5 (100%) resistant to 1 antibiotic, 3 isolates MDR; for non-dairy sites, out of 4 isolates, 4 resistant to 1 antibiotic, 2 MDR. Effect estimates and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	ARB / Detection method/ Antibiotics	Relevant findings
Li et al., (2015b)	Prevalence of resistant *Enterococcus spp.* isolated from groundwater in wells near-dairy farms (less than 2.4 km) and from wells far from the farms (non-dairy)	Low (Small spatial scale and no other likely sources influencing the comparison in that scale)	Sensititre (CLSI) / Not reported	At the sites near-dairy, out of 8 isolates, 8 resistant to 1 antibiotic (100%), 8 MDR; for non-dairy sites, 6/6 resistant to 1 antibiotic (100%), 5 MDR. Effect estimates and accompanying statistical inference were not provided
Sapkota et al. (2007)	Proportion of resistant *Enterococcus spp*. isolated from river surface water at one upstream pond (UG SW) ,about 200 m from a swine farm (SW), compared to a pool of 3 sites downstream: a site near the swine farm (DG SW1), a site 200 m downstream from it (DG SW2), and a site 100 m from SW2 (DG SW3)	Low (Small spatial scale)	Disk diffusion (CLSI) / 5 antibiotics	Proportion of resistant *Enterococcus spp.* higher downstream compared to upstream for all antibiotics, and statistically significant (Fisher's exact test) for erythromycin (p-value: 0.02), except for clindamycin (100% upstream compared to 89% downstream, p-value: 0.76). Effect estimates were not provided

Citation	Relevant comparison	Overall risk of bias	ARB / Detection method/ Antibiotics	Relevant findings
Sapkota et al. (2007)	Proportion of resistant *Enterococcus spp.* isolated from river groundwater at one up gradient well (UG GW), 300 m from SW, and at one downgradient well (DG GW), 200 m from SW	Low (Small spatial scale)	Disk diffusion (CLSI) / 5 antibiotics	Proportion of resistant *Enterococcus spp.* higher upgradient compared to downgradient for erythromycin (67% vs 20%, p-value<0.001), and vancomycin (10% vs 0%, p-value 0.15; Fisher's exact test). Proportion higher downgradient compared to up gradient for tetracycline (19% vs 3%, p-value 0.07), and clindamycin (100% vs 0%, p-value <0.001). Effect estimates were not provided
Hsu et al. (2014)	Prevalence of resistant heterotrophic bacteria isolated from river surface water at upstream sites (S1 and S2, separated by 200 m) and downstream sites (S3 and S4) from a swine farm (S2 and S3 with a separation of 400 m)	Low (Small spatial scale)	Disk diffusion (Not reported) / Sulfonamide	Prevalence was two orders of magnitude lower in upstream site S2 compared to downstream site S4. Effect estimates and accompanying statistical inference were not provided
Hsu et al. (2014)	Prevalence of resistant heterotrophic bacteria isolated from groundwater upstream (W1, between S1 and S2), near the swine	Low (Small spatial scale)	Disk diffusion (Not reported) / Sulfonamide	The highest prevalence was at W2, 3-fold higher compared to W1 (26.7% vs 9.2%), followed by the downstream well W3 (19.98%). Effect estimates and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	ARB / Detection method/ Antibiotics	Relevant findings
	farm (W2), and downstream (W3, located after S4)			
Hsu et al. (2014)	Among resistant heterotrophic isolates, the relative gene abundance in surface river water sampled at sites immediately (S3) and 400 m (S4) downstream of a swine farm compared to a site 400 m upstream (S2) of the farm. Relative gene abundance was defined: gene copies/16S rRNA gene copies	Low (Small spatial scale)	*sul1* *sul2* *sul3*	The evidence suggests a possible effect of the swine farm on relative abundance for some *sul* genes. Relative abundance of ***sul1*** and ***sul2*** was greater downstream (S3, S4) compared to upstream, however quantitative effect measures and statistical inference were not provided. The remaining gene, ***sul3***, was not detected at any site
von Salviati et al. (2015)	Proportion of ESBL/AmpC-producing *E. coli* isolated from air samples 100 m upwind and 50 m	Low (Small spatial scale)	Disk diffusion (CLSI) / 6 antibiotcs (or combinations thereof)	Qualitatively, no difference between upwind and downwind proportion of resistant isolates. Effect estimates and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	ARB / Detection method/ Antibiotics	Relevant findings
	downwind from swine barns			
Anderson et al. (2014)	Count of tetracycline resistant *E. coli* isolated from surface water at 7 upstream sites and 4 downstream sites from the effluent of a poultry processing plant at different sampling dates	Unclear (Other runoff present in the study area could impact the results but not enough information provided)	Disk diffusion (Study defined) / Tetracycline	High variability by sampling date, no clear trend. Effect estimates and accompanying statistical inference were not provided
Kerry et al. (1995)	Proportion of resistant bacteria (unspecified) isolated from sediment at fish Farm A and B and compared to sites C and D (at least 5 km away)	Unclear (Not enough information to determine potential biases)	Disk diffusion (Unspecified) / Oxytetracycline	Proportion of resistant bacteria was higher at the Farm sites A and B compared to further away (C and D). Effect estimates and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	ARB / Detection method/ Antibiotics	Relevant findings
Laube et al. (2014)	Proportion of *E. coli* harboring bla_{TEM-1} isolated from air samples 50 m downwind compared to 100 m upwind locations at 7 broiler chicken farms pooled across time points	Unclear (Other farms nearby could confound the results)	Disk diffusion (CLSI) / 5 antibiotics	Proportion of *E. coli* harboring bla_{TEM-1} was 10% and 5of air samples positive for ESBL/AmpC-producing *E. coli* on the 50 m downwind and the 100 m upwind side, respectively. Effect estimates and accompanying statistical inference were not provided
Li et al. (2015a)	Proportion of ESBL-producing *E. coli* isolated from river surface water 10 m upstream and 20 m downstream from a swine farm	Unclear (Not enough information provided)	Disk diffusion (CLSI) / 12 antibiotics	Proportion of ESBL-producing *E. coli* resistant to all 12 antibiotics was higher downstream than upstream (52.9% vs 22.2%). Effect estimates and accompanying statistical inference were not provided
Sulzner et al. (2014)	Prevalence of resistant *E. coli* isolated from cloacal samples of turkey vultures (*Cathartes aura*) near a sheep flock (HREC) compared to samples distant from sheep at a	Unclear (Not enough information about other potential exposures for the turkey vultures)	Not reported (Not reported) / 10 antibiotics	Overall prevalence of resistant *E. coli* was higher at HREC compared to LHBCR, but not statistically significant (Pearson Chi-Square; $p<0.05$). Effect estimates were not provided

Citation	Relevant comparison	Overall risk of bias	ARB / Detection method/ Antibiotics	Relevant findings
	more remote location (LHBCR)			
Gordon et al. (2007)	Proportion of resistant *Aeromonas spp.* isolated from river sediment upstream, immediately downstream from a fish farm effluent, and at a site 100 m downstream from the fish farm	High (Several other potential sources that discharge into the same river near the fish farm and no control for confounding)	Disk diffusion (CA-SFM) / 3 antibiotics	No detection of resistant *Aeromonas spp.* upstream compared to a range of 0.01 -25.9% at downstream sites (pooling both downstream sites). Effect estimates and accompanying statistical inference were not provided
Yao et al. (2011)	Proportion of resistant *Enterobacteriacea* isolated from soil within 500 m radius of a swine farm and from a site 50 km away from the farm	High (Large spatial scale with many other)	Disk diffusion (CLSI) / Amikacin	Proportion of resistant *Enterobacteriacea* was higher at the site near the farm compared to the far away site (45.2% vs 33.9%). Effect estimates and accompanying statistical inference were not provided

APPENDIX B

Table 17. Findings for the studies included in the systematic review of ARG outcomes that assessed human waste (WWTP, industrial, city) as a point source, organized by risk of bias (from low to high) (n= 19). WWTP: Wastewater Treatment Plant.

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
Amos et al. (2015)	Log-log regression model (Model 2) explaining relative abundance of *intl1* in Thames River sediment at sites across a range of WWTP impacts (defined as a function of type of, size of, and river course distance from WWTPs), adjusting for land cover, season and rainfall. Relative gene abundance was calculated as: number of *intl1*/number of 16S rRNA genes	Low (Despite large spatial scale, they adjust for potential confounders)	*intl1*	Assuming all variables included in Model 2 are independent predictors of *intl1* relative abundance, the model indicated that a 10% increase in the total WWTP impact at a given site is associated with a 3.2% increase in *intl1* relative abundance adjusting for land cover, season and rainfall (β=0.3207 $\pm$0.0723, $p<0.001$). The model predicted the impact of a large activated sludge-treatment plant on a clean site in the river to be a 200-fold (0.01-2.44%) increase in *intl1* relative abundance immediately downstream and 65-fold 10 km downstream. This model explained 83% of the variation in log *intl1* relative abundance at a single point in a river at any season within the sample used to construct the model (adjusted R^2=0.83) and 78% of the variation in a sample of four independent sites from elsewhere on the River Thames (out of sample validation)

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
Berglund et al. (2015)	Relative gene abundance in river water between sites immediately (R3), 1 km (R4) and 2.5 km (R5) downstream compared to sites immediately (R2) and 1 km (R1) upstream of a WWTP. Relative gene abundance was calculated as: ARG copies/16S rRNA copies	Low (Small spatial scale-unlikely influence from other point or non-point sources)	*dfr1* *ermB* *intl1* *sul1* *tetA* *tetB* *vanB*	Overall, relative gene abundances were higher at downstream (R3) compared to upstream (R1 and R2) sites. ***dfr1***: above detection limit downstream versus below detection limit upstream ***ermB***: relative abundance higher downstream versus upstream ($p<0.01$) ***intl1:*** relative abundance approximately 10 times higher downstream versus upstream ($p < 0.001$) ***sul1:*** relative abundance 10 times higher downstream versus upstream ($p < 0.01$) ***tetA***: relative abundance 10 times higher downstream versus upstream ($p < 0.01$) ***tetB***: above detection limit at 2 of 3 downstream sites (R3 and R4) versus below detection limit upstream ***vanB:*** no statistical difference between sites; not detected at R2
Proia et al. (2016)	On 4 rivers (designated ARB, BRE, GUA, SMP) each featuring a WWTP, relative gene abundance in biofilm from sites 50-100 m (DW) and 1 km (DW1) downstream of WWTP compared to a site 100 m upstream (UP) accounting for variation between rivers. Relative gene abundance was calculated as: ARG copies/16S rRNA gene copies	Low (Small spatial scale for each comparison, and lack of other activities that may influence the outcome is mentioned)	$bla_{CTX\text{-}M}$ *ermB* *qnrS* *sul1*	Overall, despite variation between rivers, relative gene abundance was significantly higher at downstream sites, particularly site DW, compared to upstream. ANOVA results indicated that the magnitude of this effect differed between rivers. No effect estimates were provided. Alpha cut-off for statistical inference was $\alpha=0.05$

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
Pruden et al. (2012)	General linear regression models (Model 9) explaining log relative gene abundance averaged across river sediment type (bed and suspended) and across season in a river basin as function of upstream capacities of animal feeding operations and WWTPs weighted for inverse distance along surface water pathways. Samples were collected at 10 sites along the river basin representing a range of exposure types. Relative gene abundance was calculated as: ARG copies/16S rRNA gene copies	Low (Despite a large spatial potential confounding was controlled for)	*sul1* *tetW*	The association between average log relative ***sul1*** abundance and the combined impact of inverse-distance-weighted upstream WWTP and animal feeding operation capacities was statistically significant (Model 9_{sul1}, F=40.2, p<0.0001, DF=7, R^2=0.92). Average log relative ***tetW*** abundance was not found to be associated with upstream WWTPs and animal feeding operations (Model 9_{tetW}, F=0.2, p=0.8391, DF=7, R^2=0.06). Individual effect estimates for WWTPs and animal feeding operations, respectively, and accompanying statistical inference were not provided in a fully adjusted model for either gene.
Sidrach-Cardona et al. (2014)	Relative gene abundance in river water and sediment samples at a site downstream (site 2) compared to upstream (site 1) of an antibiotic production plant (APP). Relative gene abundance was calculated as: log (ARG copies/16S rRNA gene copies)	Low	$bla_{CTX\text{-}M}$ bla_{SHV} bla_{TEM}	In surface water or sediment, graphical inspection of relative abundance means and standard errors compared between sites 2 and 1 did not support an effect of the APP on downstream gene abundance for any gene investigated. $\boldsymbol{bla_{SHV}}$ was not detected at either site in sediment. Effect estimates and statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
Sidrach-Cardona et al. (2014)	Relative gene abundance in river water and sediment samples at sites downstream (sites 4-6) compared to upstream (site 3) of a WWTP. Sites 3-6 were located 10 km downstream of an antibiotic production plant. Relative gene abundance was calculated as: log (ARG copies/16S rRNA gene copies)	Low	$bla_{CTX\text{-}M}$ bla_{SHV} bla_{TEM}	Evidence was mixed for an effect of the WWTP on downstream relative gene abundance. Graphical inspection of relative abundance means and standard errors at site 3 compared to sites 4-6 suggest that abundance of $\boldsymbol{bla_{CTX\text{-}M}}$ and $\boldsymbol{bla_{SHV}}$ in river water and $\boldsymbol{bla_{SHV}}$ in sediment may have been significantly higher downstream of the WWTP compared to upstream. But similar findings were not evident for other comparisons. Effect estimates and statistical inference were not provided
Stalder et al. (2014)	In river water at sites 3 km downstream compared to 2 km upstream of WWTP discharge point. Relative gene abundance was calculated as: ARG copies/16S rRNA gene copies	Low (Unlike influence of other sources)	*intl1* *intl2* *intl3*	No difference in relative gene abundance was observed between upstream and downstream sites ($p>0.05$) for any gene tested
Czekalski et al. (2012)	Relative gene abundance in lake water near WWTP outfall site (STEP) compared to site 3.2 km away (DP). Relative gene abundance was calculated as: ARG copies/16S rRNA copies	Unclear (Insufficient information about possible confounding due to lake depth)	*sul1* *sul2*	Qualitatively, no difference in relative abundance of ***sul1, sul2*** between STEP and DP not reported (both less than 1% in relative abundance at both sites)

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
Czekalski et al. (2014)	Relative gene abundance in lake sediment compared across a range of distances (0 to ~6 km) from WWTP outfall (STEP). Relative gene abundance was calculated as: ARG copies/16S rRNA copies	Unclear (Insufficient information about possible confounding due to lake depth)	*sul1* *sul2* *tetB* *tetM* *tetW* *qnrA*	Graphical regression analysis supported exponential decay of ***sul1, sul2, tetB, tetM,*** and ***tetW*** relative abundance at increasing distance from STEP, and interpolation analysis suggested directionality of impact. Relative ***qnrA*** abundance was below the detection limit at all sites. Statistical inference for these differences was not provided
Khan et al. (2013)	Relative gene abundance in river sediment sampled at downstream sites near (R2) and 19 km (R3) from Lahore city center compared to a site 6 km upstream (R1) from the city. Relative gene abundance was calculated as: ARG copies/10^6×16S rRNA gene copies	Unclear (Not enough information about other potential sources)	*dfrA1* *ermB* *intl1* *sul1* *tetA* *tetB*	Evidence suggested an effect of distance to Lahore city center on the relative abundance of all target genes. Although quantitative effect measures were not provided, a significant increasing trend from R1 to R3 was reported ($p < 0.01$)
Kristiansson et al. (2011)	Relative gene abundance in river sediment at sites 0.05 km (R4), 2.3 km (R2), 2.7 km (R3), and 17.5 km (R1) downstream from WWTP compared to sites located 1.9 km (R5) and 2.2 km (R6) upstream (India). Relative gene abundance was calculated in relation to the total number of identified bacterial cells	Unclear (Not enough information provided about sampling sites to determine if other sources would	*qnrS* *sul2* *strA* *strB*	Relative abundance of ***sul2*** (66 times), ***strA*** (22 times), and ***strB*** (54 times) was higher downstream compared to upstream. Relative abundance of ***qnrS*** was lower downstream compared to upstream. Effect estimates (computed differences) and accompanying statistical inference was not provided

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
		influence the outcome)		
Kristiansson et al. (2011)	Relative gene abundance in river sediment a site 25-230 m (N) downstream from WWTP compared to a site located 5-100 m (U) upstream (Sweden). Relative gene abundance was calculated in relation to the total number of identified bacterial cells	Unclear (Not enough information provided about sampling sites to determine if other sources would influence the outcome	*qnrS* *sul2* *strA* *strB*	***sul2***, ***strA*** and ***strB*** were not detected at any site. Relative abundance of qnrS was slightly higher downstream compared to upstream. Effect estimates (computed differences) and accompanying statistical inference was not provided
Lapara et al. (2011)	Gene concentration in surface water at sites approximately 1.5-24 km downstream/distant from WWTP compared to site approximately 1.6-9 km miles upstream; and gene concentration in sediment at sites near WWTP outfall compared to sites approximately 5 km and 16 km distant. Gene concentration was calculated as: ARG copies/mL water	Unclear (Not enough information about selection of sampling sites or influence of other potential sources)	*intl1* *tetA* *tetX* *tetW*	In water, concentrations of ***intl1, tetA, tetX, and tetW*** were higher immediately at the WWTP outfall, but there was little or no apparent difference between upstream and downstream/distant sites. In sediment, samples concentrations of ***intl1, tetA, tetX, and tetW*** were higher at a site near the WTTP (DH1) compared to the more distant sites (DH3 and LS1). Effect estimates (computed differences) and accompanying statistical inference was not provided

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
Makowska et al. (2016)	Mean relative gene abundance in river water at sites downstream of WWTP compared to sites upstream of WWTP. The distances from WWTP were not reported. The relative abundance was calculated: gene copies/ 16S rRNA gene × average number of 16S rRNA gene copies per bacterial cell × 100	Unclear (Not enough information about other potential sources)	*intl1* *sul1* *sul2* *tetA* *tetB* *tetM*	Mean relative gene abundances were higher at downstream sites compared to upstream sites for all genes tested, however none of these differences were found to be statistically significant. Effect estimates (computed differences) and p-values not reported ***intl1***: 0.65 (downstream) vs. 0.21 (upstream) ***sul1:*** 0.49 (downstream) vs. 0.07 (upstream) ***sul2***: 0.40 (downstream) vs. 0.17 (upstream) ***tetA:*** 0.053 (downstream) vs. 0.004 (upstream) ***tetB***: 0.053 (downstream) vs. 0.014 (upstream) ***tetM:*** 0.016 (downstream) vs. 0.009 (upstream)
Marti et al. (2013)	Relative gene abundance in sediment and biofilm samples from a site 100 m downstream compared to a site 100 m upstream of the WWTP. Relative gene abundance was calculated as: ARG copies/16S rRNA gene copies	Unclear (Not enough information about other potential sources affecting the upstream site)	*$bla_{CTX\text{-}M}$* *bla_{SHV}* *bla_{TEM}* *ermB* *qnrA* *qnrB* *qnrS* *sul1* *sul2* *tetO* *tetW*	Overall, there was evidence that the WWTP impacted the relative abundance of resistance genes in sediment and biofilm samples. Effect estimates were not provided. In sediment samples, relative abundance of ***ermB*** was significantly higher downstream compared to upstream. Relative abundance was also slightly higher downstream than upstream for most other genes (***$bla_{CTX\text{-}M}$***, ***bla_{SHV}***, ***bla_{TEM}***, ***qnrS, sul1, sul2, tetO, tetW***), but these difference were not significant. The

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
				remaining gene examined, ***qnrA***, was not detected at either river site. In biofilm samples, relative abundance was significantly higher downstream compared to upstream for most genes examined (***bla$_{TEM}$***, ***bla$_{SHV}$***, ***ermB***, ***qnrB***, ***qnrS***, ***sul1***, ***sul2***, ***tetO***, and ***tetW***) at a=0.05. And while relative gene abundance was also higher downstream compared to upstream for ***bla$_{CTX-M}$***, this difference was not significant. For ***qnrA***, relative gene abundance was lower (not detected) downstream compared to upstream, but the difference was not significant. Alpha cut-off for comparison was α=0.05
Pei et al. (2006)	Relative gene abundance in river sediment sampled at a site downstream from a city and point of discharge of a wastewater reclamation facility (site 4) compared to a site upstream from the city and discharge point (site 2). Sampling was conducted during high-flow and low-flow conditions, yielding a comparison for each sampling condition. Relative gene abundance was	Unclear (Not enough information provided about the potential influence of other point sources)	*sul1* *sul2* *tetO* *tetW*	Evidence supporting an effect of the city and discharge point on gene abundance was mixed. Qualitative comparison indicated higher mean relative abundance of ***sul1*** (during high-and low-flow conditions) and ***tetO*** (during high-flow conditions) when comparing downstream (site 4) to upstream (site 2) sites. However, there was no difference or a lower abundance downstream for ***sul1***, ***tetW*** and ***tetO*** (during high-flow conditions). Effect measures and accompanying statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
	calculated as: ARG copies/16S rRNA gene copies			
Rodriguez-Mozaz et al. (2015)	Absolute gene concentration in river water at a site 250 m downstream compared to a site 250 m upstream from a WWTP. Gene concentration was calculated as: log (ARG copies/mL water)	Unclear (Not enough information provided about selection of samples, other potential sources, or about inclusion of other covariates in the analysis)	*bla_{TEM}* *ermB* *qnrS* *sulI* *tetW*	Mean concentration of ***ermB***, ***qnrS***, and ***sulI*** was significantly higher downstream compared to upstream ($p < 0.05$). Mean concentration of ***bla_{TEM}, tetW*** was not significantly different at upstream and downstream sites ($p>0.05$).
Uyaguari et al. (2011)	Gene concentration (copies/g sediment) and relative gene abundance (copies/ng DNA) in sediment and river water samples from sites near (site 2) and downstream of (site 4) WWTP outfall compared to site upstream (site 3)	Unclear (Different type of water, river and ocean, could affect the outcome; Not enough information about	*bla_{M-1}*	Evidence did not support an increase in gene concentration or relative abundance associated with the WWTP. Both concentration and relative abundance were significantly lower near the WWTP outfall and downstream compared to upstream ($p<0.05$)

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
		potential influence of other sources)		
Xu et al. (2015)	Relative gene abundance in river water at a site downstream (T2) compared to upstream (T1) of a WWTP. Relative gene abundance was calculated as: ARG copies/16S rRNA gene copies	Unclear (Not enough information about sampling locations or other potential sources upstream or around the source)	*gyrA* *parC* *qnrC* *qnrD* *sul1* *sul2* *sul3* *tetA* *tetB* *tete* *tetM* *tetW* *tetZ*	Qualitative evidence was conflicting for an effect of the WWTP on relative gene abundance generally. Mean relative abundances of ***parC, qnrC, qnrD, sul1, tetA, tetE, tetZ*** were higher downstream compared to upstream, while the differences in relative abundance of ***gyrA, sul2, sul3, tetB, tetW*** were not significantly different between downstream and upstream sites. Relative abundance of ***tetM*** was significantly lower downstream compared to upstream. Effect estimates and accompanying statistical inference were not provided
Lapara et al. (2015)	Fluid kinetics (plug-flow) model explaining relative abundance of genes in the upper Mississippi River (>960 km reach) as a function of river flow rates, downstream distance from WWTPs, volume of fluid inputs, and modeling assumptions. Relative gene abundance was	High (Large spatial scale assessing WWTP but not considering influence of agricultural and other sources)	*bla* *ermB* *intl1* IncA/C plasmid *qnrA* *sul1* *tetA* *tetW* *tetX*	Overall, qualitative comparison of model predictions with measurements from 12 sampling locations along the river reach did not show good fit to the data for ***intl1***, ***ermB***, ***sul1***, ***tetA***, ***tetW***, and ***tetX***. **IncA/C plasmids** and a synthetic beta-lactamase (***bla***) gene were not detected in river water. Model results for ***qnrA*** were not reported. Summary effect estimates were not available due to the nonlinearity of the model

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
	calculated as: ARG copies / 16S rRNA copies			
Zhang et al. (2013)	Relative gene abundance in river water at a site downstream (N6) compared to upstream (N5) from a town. Relative abundance was calculated as: log (ARG copies/16S rRNA gene copies)	High (Many potential sources besides the one of interest, and large spatial scale)	*aacC1* *bla*$_{TEM}$ *bla*$_{OXA1}$ *cmlA5* *dfrA1* *ermB* *sul2* *tetA* *tetG* *strA* *vanA*	There was little support for an effect of the town on relative gene abundance in river water. Differences in the mean relative abundance were not qualitatively apparent for any gene tested. Effect measures (difference in means) and accompanying statistical inference were not provided for this comparison

Table 18. Findings for the studies included in the systematic review assessing ARG outcomes that had animal agriculture (both terrestrial and aquaculture) as a point source, presented by risk of bias (from low to high) (n=5).

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
Harnisz et al. (2015)	Relative gene abundance at a site approximately 200 m downstream from the discharge point of a freshwater fish farm (DRW) compared to a site approximately 200 m upstream (URW) from that point. Relative gene abundance was calculated as: log (ARG copies/16S rRNA gene copies)	Low (Small spatial scale, hence unlike influence from other sources)	*tetA* *tetC* *tetL* *tetO*	Relative gene abundance was higher downstream compared to upstream for some genes for some sampling periods, but these differences were not statistically significant ($p > 0.13$ for all comparisons)
Mceachran et al. (2015)	Relative gene abundance in airborne particulate matter collected at sites 10-20 m downwind from beef cattle feedlots compared to sites 10-20 m upwind. Relative gene abundance was calculated as: ARG copies/16S copies	Low (Confounding is addressed by weather and other feed yards by restriction, plus small spatial scale)	*tetB* *tetL* *tetM* *tetO* *tetQ* *tetW*	Evidence supports an effect of the cattle feedlot on the *tet* gene abundance in airborne particulate matter. Genes ranged from 100 to over 1,000-fold more abundant in downwind samples compared to upwind samples ($p<0.002$). The greatest relative increase was observed for ***tetM***. Statistical test used for inference was not described

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
Hong et al. (2013)	Gene abundance in groundwater at sites down-gradient from a swine farm (E4, E6, E7) compared to sites up-gradient from the farm (E1, Facility well). Gene abundance was reported as log (copies/ng DNA)	Unclear (Not enough information provided about other potential sources)	*tetZ* *tetQ* *intl1* *intl2*	Qualitative comparison of down-gradient versus up-gradient sites does not support an effect of the farm on gene abundance. Genes were detected in qualitatively similar abundance at the facility well and down-gradient sites. Genes were not detected at site E1. No effect measures or accompanying statistical inference were provided
Tamminen et al. (2011)	Relative gene abundance in marine sediment collected at varying distances (200, 400, 600, 800, and 1000 m) from a fish farm boundary on several sampling occasions over a 2 year period. Relative gene abundance was calculated as: gene copies/16S rRNA gene copies	Unclear (Not enough information provided about the sampling locations)	*tetA* *tetC* *tetH* *tetM*	There was no evidence of an effect of distance to the fish farm on relative abundance of the targeted *tet* genes in sediment. Abundances were below the limit of detection at all sites outside the fish farm boundary
Jia et al. (2014)	Relative gene abundance in surface water sampled along water course at varying distance from a swine farm. Sites S2 and S3 were along the drainage gutter at approximately 0.5 and 1 km downstream, respectively. Sites S4-S8 were along the receiving river system at approximately 2-6 km downstream. Relative gene	High (No information provided about other sources with an overall study spatial scale of 10 km)	*tetC* *tetM* *tetO* *tetQ* *tetW* *tetX*	Qualitatively, evidence suggested a sigmoidal decay of gene abundance at increasing distance from the swine farm supporting a possible effect of the farm on gene abundance in the waterway. In particular, relative abundance was higher in the drainage gutter compared to the river. However, effect estimates and statistical inference were not provided

Citation	Relevant comparison	Overall risk of bias	Gene(s)	Relevant findings
	abundance was calculated as ARG copies/16S rRNA copies			

APPENDIX C

Table 19. Complete list of the gene array with the antibiotic resistance genes (ARG), primer sequences (forward and reverse), and references used for studies in Chapters 4 and 5.

Gene Name	Forward (5'→3')	Reverse (3'→5')	Reference
16S rRNA	CCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG	Muyzer et al., 1993
aacA	GTGTAACACGCAAGCACGAT	AGCCTCCGCGATTTCATAC	Szczepanowski et al., 2009
aadA5	ATCTTGCGATTTTGCTGACC	TGTACCAAATGCGAGCAAGA	Szczepanowski et al., 2009
ampC	CCTCTTGCTCCACATTTGCT	ACAACGTTTGCTGTGTGACG	Szczepanowski et al., 2009
bla_{KPC}	GATACCACGTTCCGTCTGG	GCAGGTTCCGGTTTTGTCTC	Hindiyeh et al., 2008
bla_{NPS}	GGACCATCGTCATCGAGTCT	ATTCGCAATCGAATACTGGG	Szczepanowski et al., 2009
bla_{OXA}	TGATGATTGTCGAAGCCAAA	GCCTGTAGGCCACTCTACCC	Ross et al., 2015
bla_{SHV}	AACGGAACTGAATGAGGCGCT	TCCACCATCCACTGCAGCAGCT	Chia et al., 2005
bla_{VIM}	CGCAGCTTTCTGGTTGGTAT	CGTGTCACCGAGTTTCTGAG	Szczepanowski et al., 2009
bla_{CMY}	ACTCCGGGCGCTAAGCGACTTTAC	CGCCAATACGCCAGTAGCGAGAC	Johnson et al., 2011
bla_{CTX}	AGCGGCAGTCGGGAGGCAGAC	GCCCGGAATGGCGGTGTTTA	Johnson et al., 2011

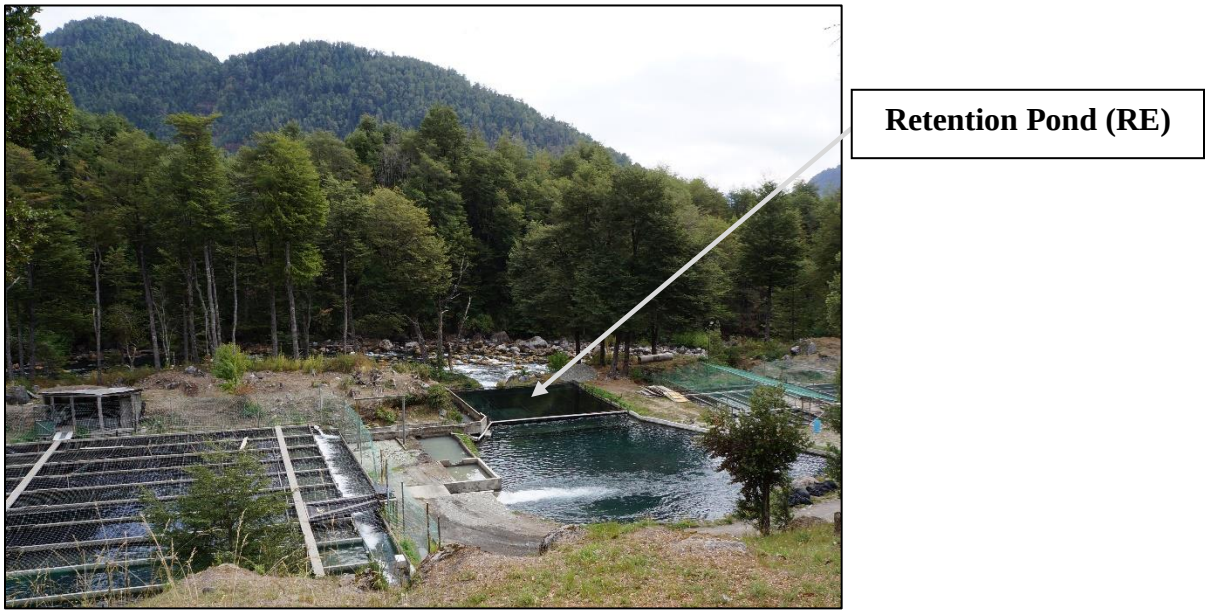
Gene Name	Forward (5'→3')	Reverse (3'→5')	Reference
***bla*$_{IMP}$**	AAGTTAGTCAMTTGGTTTGTGGAGC	CAAACCACTACGTTATCTKGAGTGTG	Calderaro et al., 2017
***bla*$_{NDM-1}$**	TGACGCGGCGTAGTGCTCAGTGT	GCGGCGGGGATTGCGACTTAT	Johnson et al., 2011
***bla*$_{PER-2}$**	CCGTGGTAGCAAATGAAGCG	ACCGGTTTTATGCGCCACTA	Johnson et al., 2011
***bla*$_{TEM}$**	CCGTGTCGCCCTTATTCCCTTTTT	GCTCTTGCCCGGCGTCAACAC	Johnson et al., 2011
dfr13	AATCGGTCCGCATTTATCTG	TTGGTAAGGGCTTGCCTATG	Szczepanowski et al., 2009
ermB	GATACCGTTTACGAAATTGG	GAATCGAGACTTGAGTGTGC	Chen et al., 2017
ermF	CGACACAGCTTTGGTTGAAC	GGACCTACCTCATAGACAAG	Ma et al., 2011
floR	TCGTCATCTACGGCCTTTTC	CTTGACTTGATCCAGAGGGC	Szczepanowski et al., 2009
intl1	CCTCCCGCACGATGATC	TCCACGCATCGTCAGGC	Goldstein et al., 2001
intI2	GACGGCTACCCTCTGTTATCTC	TGCTTTTCCCACCCTTACC	Barraud et al., 2010
intI3	GGATGTCTGTGCCTGCTTG	GCCACCACTTGTTTGAGGA	Barraud et al., 2010
mcr-1	ACACTTATGGCACGGTCTATG	GCACACCCAAACCAATGATAC	Bocanegra-Ibarias et al., 2017
mecA	AAAAAGATGGCAAAGATATTCAA	TTCTTCGTTACTCATGCCATACA	Szczepanowski et al., 2009
mecC	GCAAGCAATAGAATCATCAGACAA	CGATTCCCAAATCTTGCATACC	This study

Gene Name	Forward (5'→3')	Reverse (3'→5')	Reference
qacG	TGGTTATTTCTGGCTACGGC	TTTGAGTGTCAGCGACAGGA	Cummings et al., 2010
qnrB	AAATATGGCTCTGGCACTCG	CTTTCAGCATCGCACGACTA	Szczepanowski et al., 2009
qnrS	GACGTGCTAACTTGCGTGAT	TGGCATTGTTGGAAACTTG	Marti et al., 2013
strB	CGCAGTTCATCAGCAATGTC	GCCTGTTTTTCCTGCTCATT	Szczepanowski et al., 2009
sul1	CCGTTGGCCTTCCTGTAAAG	TTGCCGATCGCGTGAAGT	Heuer et al., 2007
sul2	GACAGTTATCAACCCGCGAC	GTCTTGCACCGAATGCATAA	Szczepanowski et al., 2009
sul3	TCCGTTCAGCGAATTGGTGCAG	TTCGTTCACGCCTTACACCAGC	Pei et al., 2006
tetA	GCTACATCCTGCTTGCCTTC	CATAGATCGCCGTGAAGAGG	Ng et al., 2001
tetB	AGTGCGCTTTGGATGCTGTA	AGCCCCAGTAGCTCCTGTGA	Looft et al., 2012
tetC	TGTTTCGGCGTGGGTATG	CATTAGGAAGCAGCCCAGTAG	This study
tetL	TCGTTAGCGTGCTGTCATTC	GTATCCCACCAATGTAGCCG	Ng et al., 2001
tetM	GTGGACAAAGGTACAACGAG	CGGTAAAGTTCGTCACACAC	Ng et al., 2001
tetQ	CGCCTCAGAAGTAAGTTCATACACTAAG	TCGTTCATGCGGATATTATCAGAAT	Looft et al., 2012
tetS	CAAGGATTGTACGGTTGGAAA	TTTCGAAGCTAAGATATGGCTC	Szczepanowski et al., 2009

Gene Name	Forward (5'→3')	Reverse (3'→5')	Reference
tetW	GAGAGCCTGCTATATGCCAGC	GGGCGTATCCACAATGTTAAC	Aminov et al., 2001
tetX	AGCCTTACCAATGGGTGTAAA	TTCTTACCTTGGACATCCCG	Ghosh et al., 2009
vanA	GTAGGCTGCGATATTCAAAGC	CGATTCAATTGCGTAGTCCAA	Bell et al., 1998
vanB	TTGCATGGACAAATCACTGG	GCTCGTTTTCCTGATGGATG	Graham et al., 2008
vatB	GGAAAAAGCAACTCCATCTCTTGA	TCCTGGCATAACAGTAACATTCTGA	Looft et al., 2012
vatC	CGGAAATTGGGAACGATGTT	GCAATAATAGCCCCGTTTCCTA	Looft et al., 2012
vatE	GACCGTCCTACCAGGCGTAA	TTGGATTGCCACCGACAATT	Looft et al., 2012
vgbB	CAGCCGGATTCTGGTCCTT	TACGATCTCCATTCAATTGGGTAAA	Looft et al., 2012

Figure 22. Raceway structure at one of the trout farms.

Figure 23. One of the trout farms showing the location of the retention pond (RE).



APPENDIX D

Table 20. Coefficients of linear discriminants for both the absolute and the relative scales from linear discrimination analysis for bird and water samples (in bold the genes that influenced the most each LD).

	Absolute scale (Relative scale)	
Gene	**LD1**	**LD2**
***bla*$_{SHV}$**	0.45 (-0.43)	**0.81 (1.06)**
***bla*$_{CTX}$**	-0.26 (-1.67)	-0.63 (-1.19)
***bla*$_{TEM}$**	-0.99 (-1.30)	-0.10 (-0.34)
sul1	**3.46 (2.15)**	**2.88 (2.58)**
sul2	**1.19 (1.05)**	-2.92 (-1.73)

Table 21. Group means (average abundance of each gene) for each one of the sample types (Migratory, Resident, and Water). Means are expressed in absolute and relative abundance in linear scale.

Absolute scale (Relative scale)			
Gene	**M**	**R**	**W**
$\boldsymbol{bla_{SHV}}$	0.65	0.58	5.50
$\boldsymbol{bla_{CTX}}$	0.62	0.63	5.89
$\boldsymbol{bla_{TEM}}$	0.39	0.35	34.68
sul1	0.33	0.29	60.25
sul2	0.30	0.43	54.96

Figure 24. Aeration pond (i.e. biological reactor) at one of the wastewater treatment plants from the study (W3).